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PATHOGENESIS OF BLACK QUARTER

II. Influence of Nutrition and of Muscular Exercise

By

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INTRODUCTION

In a previous paper (Minett, 1948) it was shown that if yearling sheep at roughly the same stage of development are injected intramuscularly or subcutaneously with *Cl. chauvoei* spores there is great variation in susceptibility. The difference is so marked that some may promptly develop the disease and die, while others inoculated with even larger numbers of spores from the same suspension show no reaction. At the same time, it was ascertained by the use of tissue depressants (mainly calcium chloride) or by recovery of the organism after slaughter that sheep which had failed to react might continue to harbour the spores in a latent state for periods up to 25 days, in one case up to 40 days. The factors responsible for the varying susceptibility might be (*a*) a naturally acquired immunity; (*b*) a varying degree of tissue damage, *e.g.*, amount of bleeding, at the site of inoculation; (*c*) exposure to inclement weather resulting in chilling; and (*d*) the nutritional state of the animal. The first factor seemed unlikely, and may be dismissed for the moment in view of the origin and history of the animals used in this work and on the results of a few serum-neutralisation tests. Experiments made *in vitro* and *in vivo* with spores suspended in blood clot did not indicate, as has been suggested, that this medium facilitated germination, whilst information on chilling effects will be set out in the next paper. Altogether, the fourth factor, *viz.*, the nutritional state, seemed the most likely for the following reasons. It is the better-conditioned animals which are the most frequent victims of black quarter. The ease with which germination of latent spores can be set up experimentally by tissue depressants suggests that in nature germination may be brought about through tissue damage. The natural damaging agent *might* be lactic acid, liberated in excess from the higher glycogen reserve of well-nourished muscles. Möller (1911) has shown that by feeding guinea-pigs on food rich in sugar (sugar beet) their susceptibility to black quarter by inoculation (route not stated) is increased, while it is decreased by starvation or exercise. The decreased susceptibility of the starved animals is shown by the fact that some of them survive, while in those which die the characteristic appearances of black quarter are much less pronounced. In this paper similar experiments with sheep are reported.

EXPERIMENTAL

The experiments in principle were quite simple. It was hoped to bring out a difference in susceptibility by taking at random young sheep of about the same age and of mature development but rather

impoverished condition and dividing them into two groups, one of which would be put on a higher plane of feeding than the other. After a suitable period, both groups would be inoculated with a certain number of black quarter spores. Some uninoculated sheep were set aside for slaughter so that glycogen, and in some cases lactic acid, estimations might be made with their muscular tissue.

The work was carried out at Mukteswar between 1942 to 1944, using sheep from the neighbouring hills or adjacent plains. There is no record of black quarter occurring naturally in sheep in these areas, so the question of specific immunity was unlikely to arise. The spore suspensions were prepared in distilled water, as described in the previous paper, and 1 c.c. of a suitable dilution was injected into the muscle at the back of one thigh. The number of spores thus inoculated was estimated from the probability tables of Halverson and Ziegler (1933), using five tubes of meat-liver medium for every dilution tested. As regards the diets, it was anticipated that the sheep fed at the higher nutritional level would slowly increase in weight through an improvement of their muscular condition and that the controls on the lower diet would roughly maintain their body weights without showing any appreciable change in condition. An impression of the muscular condition was gained by palpation of the muscle at the back of the thigh at the spot where the spores were to be inoculated. Actually, as will be seen, the intentions were not fully realised in spite of the diet sheets being drawn up on a scientific basis. This was perhaps due in part to some of the desired ingredients not being available, so that substitutions had to be made ; in addition, there was a certain wastage from various causes among the experimental animals.

INFLUENCE OF DIET

1942

The intention was to raise the condition of the muscles and their glycogen level by increased carbohydrate feeding in the form of molasses. In August, 25 male sheep, mostly less than 18 months of age, were divided into two groups of 13 and 12 in such a way that the individuals of the two groups roughly corresponded and the mean weights were the same. The diets were as follows :

Group I (control sheep on maintenance ration) received (i) a scheduled quantity of a concentrate mixture made up of crushed oats, linseed cake and wheat bran in the proportion 7 : 2 : 1 ; and (ii) oat straw fed to appetite.

Group II (sheep on high carbohydrate diet) received (i) a scheduled quantity of a concentrate mixture made up of crushed oats, linseed cake and wheat bran in the proportion 6 : 3 : 1 ; (ii) 0·75 lb. molasses ; and (iii) oat straw fed to appetite.

As the roughage prescribed was the same, the nutritional difference in the ration for the two groups had to be brought about by manipulating the composition in such a way that the nutritive ratio

of the concentrate mixture to be fed to Groups I and II would be about 1 : 6 and 1 : 12 respectively. The widening of the ratio in the latter case was effected by the molasses supplement, rich in easily assimilable carbohydrate. In actual feeding during the course of the experiment, however, these ratios could not be rigidly maintained but were eventually adjusted at about 1 : 6 and 1 : 9 respectively.

If the sheep in Group I gained weight, the amount of concentrates was to be slightly decreased. The experimental feeding was started on August 15th with certain modifications. Since linseed cake was not available, mustard cake was substituted while green leaves were given instead of straw. At first, the sheep did not take the molasses well, tended to suffer from diarrhoea, and lost weight severely. By cutting down the amount and then slowly increasing it again, they gradually became accustomed to it, and 27 days later they were consuming $\frac{3}{4}$ lb. daily. At the same time, there was a steady rise in weight so that when the inoculations were commenced the mean weight of the animals in Group II was greater by 10 lb. On the other hand, the mean weight of Group I had declined by 7 lb. The spore inoculations were started on October 20th. The two sheep selected for injection on any one day were as alike as possible in weight and had been rested in the shed for three days before inoculation. Apart from this, from September 17th the sheep got walking exercise for one hour morning and afternoon. The results are shown in Table I. This experiment in some respects was not very satisfactory, but there is no evidence of a greater susceptibility among the sheep fed excess carbohydrate.

On October 22nd all the available sheep were tied up for three days and then made to trot for 20 minutes to see if any signs of myoglobinuria would develop, but no abnormality was seen and the urine which could be collected from most of them showed no change in colour.

TABLE I

INOCULATION OF *Cl. chauvoei* SPORES IN WELL-FED AND POORLY-FED SHEEP

Year	*Days on changed diet*	*Estimated No. of spores inoculated*	*Sheep No.*	*Body weight (lb.) at inoculation*	*High (H) or Low (L) diet*	*Fate (D=Died, hrs.) (S=Survived)*	*Remarks*
1942	66	32,000	11	51	H	S	
			12	51	L	D 36–48	
	71		13	45	H	S	
			14	47	L	S	
	74	64,000	15	51	H	S	
			16	50	L	S	
	77	160,000	17	44	H	D 48	
			18	42	L	D 36–48	
	80	96,000	9	45	H	S	
			19	40	L	S	

TABLE I—*continued*

Year	Days on changed diet	Estimated No. of spores inoculated	Sheep No.	Body weight (lb.) at inoculation	High (H) or Low (L) diet	Fate (D=Died, hrs.) (S=Survived)	Remarks
	83		20	47	H	D 72–96	
			21	43	L	S	
		128,000					
	89		22	52	H	S	
			23	40	L	D 36–48	
1943	96	240	54	60	H	S	Both good muscle
			55	50	L	S	
	66		56	47	H	D 38	Both good muscle
	98		57	46	L	S	
		2,400					
	101		58	54	H	S	
			59	48	L	S	
	104		60	50	H	D 36	Slightly better
	72		61	47	L	S	muscle than 61
		240,000					
	106		62	59	H	D 60	Both good muscle,
	74		63	50	L	D 36	slightly better in 62
		(All the above sheep, except No. 13, were males)					
1944	102	1,000	76	40	H	D 28	
			77	48	L	D 29–39	
	106	100	78	54	H	S	78 had better
			79	55	L	S	muscle
	107		80	49	H	D 36	
			81	48	L	D 47	
		550					
	112		82	52	H	S	83 muscle very
			83	35	L	S	poor
	114		84	50	H	D 45	85 muscle very
			85	45	L	D144	poor (footnote)
		5,500					
	116		86	51	H	S	87 muscle very
			87	38	L	S	poor
1944	125	5,000	88	42	H	D 24	88 muscle much
			89	44	L	D 36	better
			90	50	H	S	90 a muscular
	142	5,000	91	45	L	S	sheep, others
			92	39	L	D 53	poor
	146		93	50	H	D 52	
			94	41	L	S	
		7,000					
	149		95	49	H	D 32	
			96	40	L	D 22	
	156	6,000	97	42	H	D 29–33	97 had good
			98	44	L	S	muscle and 98 poor

Sheep 84 had a good-sized lesion, that in Sheep 85 was much smaller. On cutting the latter there was slight haemorrhagic oedema in the subcutaneous tissues and gas in the adjacent muscles, showing clostridia microscopically.

Between October 22nd and 30th, two good sheep (Nos. 24 and 25) and two poor sheep (Nos. 26 and 27) were rested for six days, then slaughtered and estimations made for glycogen and lactic acid in the muscle and lactic acid in the blood. The results, given in Table II, show only slightly lower glycogen values for the poor sheep and no consistent difference in muscle or blood lactic acid.

TABLE II

ESTIMATIONS OF MUSCLE GLYCOGEN AND OF MUSCLE AND BLOOD LACTIC ACID IN SHEEP

Year	*Sheep No.*	*Body weight (lb.) shortly before death*	*High or low diet*	*Days on changed diet*	*Glycogen mg. per 100 g. muscle*	*Lactic acid*	
						Mg. per 100 g. muscle	*Mg. per 100 c.c. blood*
	3	—	—	—	699	79·3	12·61
	5	—	—	—	420	178·0	21·6
	4	—	—	—	560	107·1	18·45
	64	—	—	—	960	147·7	11·25
	65	—	—	—	560	127·5	20·6
1942	24	46	High	76	1,452	28·9	20·3
	25	58	„	71	1,176	23·3	20·3
	*9	46	„	87	764	31·0	7·4
	*22	52	„	97	974	82·9	8·44
	26	51	Low	74	1,139	46·5	21·6
	27	43	„	68	1,069	27·3	20·9
1943	66	50	High	83	1,030·0	66·2	13·5
&	67	50	„	90	1,214·0	80·7	10·1
1944	*54	60	„	99	932·2	43·3	7·4
	68	60	„	80	846·0	39·4	12·1
	*82	52	„	146	621·7	—	—
	*86	51	„	187	1,008·5	—	—
	*90	50	„	193	1,055·0	—	—
	99	72	„	204	1,142·9	—	—
	100	61	„	211	1,212·2	—	—
	69	42	Low	86	142·0	55·9	14·8
	70	40	„	93	800·0	81·3	10·1
	*55	50	„	102	724·9	32·5	7·8
	*61	47	„	75	458·0	31·3	8·8
	71	54	„	83	714·0	107·3	9·8
	72	46	„	87	267·0	20·0	11·8
	*83	35	„	146	55·1	—	—
	*91	45	„	187	735·4	—	—
	*94	41	„	193	1,035·0	—	—

All the above were uncastrated males.

* Had been previously injected with black quarter spores and had survived.

Estimations of Sheep 3 to 65 were preliminary ones and the sheep were not specially dieted or rested.

1943

Two groups of young sheep, ten in each group, were formed in April. Most of them were males and all were estimated to be 12

to 18 months old. From May 4th, the sheep were put on the experimental diets, and in this case attention was paid to the protein level. The diets were as follows :

Group I (control sheep on maintenance ration) received (i) a scheduled quantity of a concentrate mixture made up of crushed oats, linseed cake and wheat brain in the proportion 6 : 3 : 1 ; and (ii) oat straw fed to appetite.

Group II (sheep on high protein diet) to receive (*i*) a scheduled quantity of a concentrate mixture made up of crushed oats, groundnut cake and wheat bran in the proportion 4·5 : 4·5 : 1 ; and (ii) oat straw fed to appetite.

As in 1942, the roughage prescribed remained the same. Hence the nutritional difference in the ration for the two groups had to be brought about by manipulating the composition so that the concentrate mixture to be fed to Groups I and II would yield nutritive ratios of about 1 : 5 and 1 : 2 respectively. The narrow nutritive ratio in the latter case was effected by incorporating in the mixture groundnut cake rich in protein and by reducing the proportion of crushed oats.

As before, it was necessary to modify the diet by replacing oat straw with oak leaves, and again the object was to keep Group I at steady weight and to allow sheep of Group II to increase. Both groups, however, showed some increase, though the mean weight of Group II animals was always 1½ to 3 lb. above that of Group I.

The spore inoculations were started on August 8th. An attempt was always made to choose pairs of sheep having similar muscular development as judged by digital examination at the thigh. At the time of injection the sheep were gently carried out of their loose box and after injection kept quietly tied up for some time. The results (Table I) show that in two out of three experiments, the high-protein sheep reacted and the low-protein sheep did not. In the third experiment, both reacted but both had good muscular development in spite of the different rations. This experiment suggests that high-protein feeding is more likely to increase susceptibility to black quarter spores than high carbohydrate feeding.

1944

The object of these experiments was to show the effect of a high-protein diet. Two groups of sheep, 15 in each and aged one to two years, were assembled at the beginning of April. The concentrate mixtures and roughage fed were the same as in the previous year ; experimental feeding was started on April 9th. The amount of concentrates fed was altered from time to time in accordance with body weight and muscularity. The endeavour this time was to produce a marked difference in the muscular condition of the two groups by adjusting the plane of feeding. During the first ten weeks the mean body weights of the group were almost the same, after

that the mean weight of the well-fed group was in excess by 2 lb., rising to 6 lb.

The spore inoculations were started on July 20th and continued at intervals until October 6th, as sheep in suitable condition became available. Thus, ten pairs of sheep were inoculated with equivalent numbers of black quarter spores. The results (Table I) showed that with three pairs (106, 112 and 116 days of feeding), both sheep survived ; with five pairs (102, 107, 114, 125, 149 days of feeding), both sheep died ; in four of these five pairs, death was earlier in the high-protein sheep, usually by a matter of 12 hours and in one case by four days ; with two pairs (146 and 156 days of feeding), the high-protein sheep died and the low-protein one survived.

In one further experiment (142 days of feeding), one high-protein and two low-protein sheep were used. One of the latter died of black quarter rather late (after 53 hours and with atypical lesions, *viz.*, slight oedema round the hock joint and without change at the site of inoculation ; the other two survived.

Miscellaneous

Apart from the above *ad hoc* experiments, results are available of six sheep which, although not specially fed, were noticeably different in muscular development at the time when for other purposes they were inoculated intramuscularly with spores.

(*a*) Sheep 48 and 49, yearling males, were each given 3,500 spores. Both died of black quarter ; No. 48, in good muscular condition, after 36 hours, and No. 49, a small thin sheep, after 51 hours. (*b*) Sheep 50 and 51, 12 to 18 months, male and female respectively, were each given 245,000 spores mixed with $CaCl_2$. Sheep 50 died of acute black quarter after 30 hours, while sheep 51 survived for six days. The latter had shown a transient oedema of the hock and metatarsus of the inoculated limb and gave birth to a lamb 48 hours after inoculation. Sheep 50 was in good muscular condition, while the thigh muscle of sheep 51 was quite wasted. (*c*) Sheep 52 and 53 were injected intramuscularly with equivalent numbers of spores. No. 52 was noted at the time of inoculation to be in appreciably better condition and died of black quarter after two days. Sheep 53 died after three days. Symptoms were definite 24 hours after inoculation in No. 52, but entirely absent in No. 53.

GENERAL RESULTS

Summarising the results of 1943 and 1944, and of the six sheep just mentioned, and excluding instances where both sheep of the pair survived, there were 13 pairs of sheep. Of these, in four instances, the high-diet sheep died of black quarter and the low-diet sheep survived ; in seven instances, the better-nourished sheep died earlier than its poorly-nourished companion ; in two instances, the low-diet sheep died earlier than the high-diet sheep.

Glycogen and lactic acid determinations

Glycogen and lactic acid were estimated in muscle from the back of the thigh and lactic acid was also estimated in the blood. Except the first five sheep shown in Table II, the animals had been rested for at least three days before they were killed and they were carried a few yards to the place of slaughter so that there should be as little muscular movement as possible. They were then laid on their sides, stunned, the neck vessels severed and blood for lactic acid estimation taken into citrate. Within a minute or so the skin over the back of the thigh was reflected, the muscles cut across and pieces the size of a hazel nut, always from the same region, promptly removed to 30 per cent. KOH contained in a stoppered tube in ice for glycogen estimation and into 2 per cent. HCl well-cooled in freezing mixture, for lactic acid estimation.

Glycogen was estimated according to Good *et al.* (1933). The weight of tissue was determined by weighing the tube with KOH before and after collecting the tissue. More KOH was then added to make 2 c.c. of KOH per gram of tissue taken. The tube was fitted with a condenser and heated in a boiling water-bath until the tissue dissolved completely : 1·2 volumes of 95 per cent. alcohol were then added to precipitate glycogen, which was hydrolysed by heating with 0·6 N HCl for 2 to 2½ hours. Sugar so formed was estimated by the Somogyi-Shaffer-Hartmann method. as described by Hawk and Bergeim (1938).

Lactic acid in muscle was estimated by the method of Bertho and Grassmann (1938), protein being removed according to Cori (1925). The weight of the tissue was found as above by weighing the tube with 2 per cent. HCl before and after putting the tissue into it. The tissue was then chopped and ground in a cool mortar with washed sea-sand as quickly as possible before precipitating with mercuric chloride. Sugars in the filtrate were removed by adding copper sulphate and calcium hydroxide. Lactic acid was estimated in the filtrate by converting it to aldehyde, which is bound by potassium metabisulfite, and titrating the bound sulfite iodometrically after being liberated by addition of sodium bicarbonate. Each c.c. of N/100 iodine solution used up in the titration of bound sulfite is equivalent to 0·45 mg. lactic acid.

Lactic acid in blood was also estimated according to Bertho and Grassmann (1938). Blood was deproteinised with freshly-precipitated tungstic acid, and sugars were removed from the filtrate with copper sulphate and calcium hydroxide before estimating lactic acid. The values found are shown in Table II. The figures for muscle glycogen of sheep 66 to 94 have been analysed, and the difference between the means for the nine high-protein (four in 1943 and five in 1944) and the nine low-protein sheep (six in 1943 and three in 1944) is found to be highly significant. Thus, means for the nine high and the nine low protein sheep are 1006·94 and 547·93

respectively, difference = 459·01. S.E. of the difference = 127·68.

$$t = \frac{\text{difference between means}}{\text{S.E. of difference}} = 3{\cdot}595$$

Degrees of freedom = 16

P = <0·01

Thus while the difference in muscle glycogen is significant in the high and low protein experiments, there is no such difference with the carbohydrate experiment though, of course, the number of sheep is very small. In the high and low protein experiments there is likewise no difference in the mean values for muscle or blood lactic acid.

Effect of muscular exercise

Since sheep on high concentrate feed intended to provide an abundance of protein, appear from the foregoing work to be more susceptible to experimental black quarter, and since this nutritive state is accompanied by higher muscle glycogen, it was of interest to enquire if the lactic acid that might be liberated in excess in the muscles of such sheep by exercise, would so damage the muscle as to allow spores to germinate. The case in fact might have some analogy with equine myoglobinuria in which there is often severe muscle injury and death in well-fed horses brought out for work after a few days' rest. A hypothesis in the contrary sense would be that as the working of muscle raises its oxygen tension and increases lymph flow, the chance of spores germinating would be lessened. The few experiments on the subject (see Table III) were inconclusive.

The experiments of 1942 were carried out at Mukteswar ; those of 1943 and 1945 at Izatnagar. Sheep, in pairs, were inoculated intramuscularly at the back of the thigh with a number of spores estimated to be small enough to infect some sheep only. One of the pair was then immediately exercised and the other kept at rest. The sheep used for these experiments were kept in a small pen and grazing was somewhat restricted so that the amount of muscular exercise might be limited. This was because in untrained muscles when subjected to exercise, lactic acid is said to accumulate to a higher extent than in muscles in regular training. Sheep to be used for inoculation were selected a few days in advance and were then either kept tied up or not let out for grazing. It was judged necessary in this way to do everything possible up to the moment of inoculation to minimise breakdown of muscle glycogen. The two sheep selected were usually of about equal weight and it was attempted in particular to select ones with similarly developed thigh muscles, although this was not easy and often one sheep was in appreciably better condition than the other. In the 1945 experiment, a special effort was made to use well-nourished sheep. For this purpose, 20 sheep were fed for five months on a concentrate diet (similar to mixture 2 of 1943) at $\frac{3}{4}$ to 1 lb. daily and selections made as required.

TABLE III

INOCULATION OF *Cl. chauvoei* SPORES IN SHEEP AT REST OR EXERCISED

Date	*Sheep No.*	*Body weight (lb.) at inoculation*	*No. of spores inoculated*	*Exercised (E) or Rest (R)*	*Fate (D=Died, hrs.) (S=Survival)*	*Remarks*
1942						
Nov. 16	28	72	20,000	E	D 34–40	Both sheep had been fed molasses (Group 3 of 1942 experiment)
	29	72		R	D 48–64	
„ 17	30	38	20,000	E	D 43	No molasses given (Group 1 of 1942 experiment)
	31	55		R	S	Molasses-fed sheep
Dec. 14	32	33	20,000	E	D 24	
	33	38		R	D 30–44	Rather more muscular than 32
„ 21	34	32	10,000	E	D 36	Rather less muscular than 35
	35	38		R	S	Exercised 7 days later
1943						
Jan. 28	59	—	130	E	S	
	55	—		R	S	
„ 30	73	—	1,300	E	S	
	74	—		R	S	
Feb. 1	70	—	13,000	E	S	
	75	—		R	D 48	
1945						
Oct. 11	101	46	1,500	E	S	Exercise repeated 3 hours after inoculation.
	102	47		R	S	
„ 14	103	41	7,500	E	S	
	104	41		R	D 78–94	
„ 19	105	36	7,500	E	D 48	
	106	33		R	S	
„ 23	107	42	7,500	E	S	
	108	41		R	D 72	More muscular sheep than 107
„ 31	109	44	7,500	E	D 48	
	110	41		R	S	

In this year also, a single tube of spore suspension was used throughout. The exercise given always consisted of brisk walking or trotting on the level for 30 to 60 minutes, with occasional rest periods. The companion sheep was held at rest for one to three hours. In the 1943 experiments, the latter was then transferred to a small crate where it was more or less immobilised until the next day.

In the absence of suitable apparatus for electrical stimulation as a means of inducing repeated muscular contraction, a test was made with strychnine as a means of tetanising muscle, but was not successful. A sheep weighing about 50 lb. was given 0·005 g. of

strychnine subcutaneously with only a slight effect ; two days later it was given twice this dose but death followed from strychnine poisoning within half an hour. On two occasions, a sheep was given the same number of spores into the muscles at the back of each thigh. One leg was then vigorously exercised by alternate flexion and extensions for three periods of five minutes with rest intervals of ten minutes, while the other leg was held at rest. In the first experiment, in which 1,300 spores were injected at each site, the result was negative. The persistence of spores, however, was shown by an injection of calcium at site of inoculation three days later. In the second experiment, in which 130,000 spores were given at each site, a black quarter lesion was first noticeable in the rested limb, and the animal died after 28 hours.

Summarising the results shown in Table III, it is seen that 12 experiments were carried out. In three of these, neither sheep developed black quarter ; in two, both sheep were affected, the interval before death being rather shorter in the exercised sheep ; in four experiments, the exercised sheep died of black quarter between 36 and 48 hours after inoculation, while the resting sheep were unaffected ; and in three experiments, the resting sheep succumbed to black quarter while the exercised sheep were unaffected.

DISCUSSION

There is now a considerable literature on the influence of the nutritional state on susceptibility to infections. Apart from specific nutritional deficiencies or excesses, it is known that the general bodily condition has a modifying effect on receptivity to certain infections, and on response to allergic reagents. From field observation it has been stated that this is true of several clostridal infections, including black quarter, and the experiments with sheep set out above give some support to the idea. In order to show differences in susceptibility, it is necessary that the number of spores injected should be adjusted with some care and that differences in the nutritional state of the animals should be sufficiently pronounced. As mentioned, it has not been easy to regulate matters in this respect by giving dietaries which are correct on theoretical grounds, and in 1944 chief reliance was placed on the state of the thigh muscles as judged by digital palpation. Even this is a somewhat crude method of estimating " condition." The 1942 molasses-feeding experiment was not very satisfactory, perhaps because the excessive carbohydrate feeding depressed protein digestibility. Although the richer rations given in 1943 and 1944 were intended to provide extra protein, they were also no doubt providing more carbohydrate and other nutrients. The richer ration during these two years at any rate had the effect of raising the level of muscle glycogen. Further experiments would be required to determine whether any particular constituent of the diet is more likely to influence susceptibility.

An explanation is required as to the manner in which a higher nutritional state of the muscle influences the germination of spores and multiplication of the bacilli. On the basis of Fildes' work, it can of course be accepted that the essential for germination of anaerobe spores is a sufficiently low oxygen tension. Moreover, Fildes (1927) has shown with *Cl. tetani* that this lowering of the oxygen tension need last only a very few hours to be effective; as early as two hours after ligature of the spermatic cord germination of tetanus spores injected at the same time into the testis was well under way and even before any degeneration of the tissues was observable histologically. Fildes has also pointed out that phagocytosis rather than assisting the defences may assist the invader, in that an accumulation of macrophage cells undergoing necrosis can provide conditions suitable for spore germination. In the experiments recorded in the present paper the initial injury, which was the passage of a medium-sized hypodermic needle, was minimal, and the same both in the poorly-fed and the well-fed animals. Nevertheless, the effects may well have been quantitatively different in the two sets of animals. In the well-fed group, with more highly vascularised muscle there would be a larger area of necrosis, greater bleeding and a more prolific output of macrophages to restore the damage; in other words, a relatively larger lesion or area of low oxygen tension.

An added incentive in both groups to phagocyte migration would be the spores themselves, especially as they were injected in numbers near the borderline of infectivity. It may finally be noted that in any case the oxygen tension of resting muscle is low (Campbell, 1924–25). Once germination has been established, multiplication of the organisms would be assisted by the ready supply of an easily fermentable nutrient derived from the glycogen of the well-fed muscle. This was the point made by Möller (1911) from his experiments with guinea-pigs and it has been corroborated here with sheep. Möller, however, was only concerned with what is really the second phase in the pathogenic effect of the organism. The problem that still remains is what are the factors in nature which are liable to produce in unwounded muscle the ischaemic condition necessary for the germination of spores of pathogenic anaerobes that happen to be present. Detailed speculation at this stage would serve little purpose, but a few ideas may be helpful. Dr. S. N. Ray has suggested to me that the higher synthesis of vitamins which presumably accompanies the higher dietary intake may induce greater enzyme activity and a sufficient lowering of oxygen tension may thus be accounted for. One may put forward other hypotheses. It may be that in nature slight localised inflammations, with or without accumulations of phagocytes, are prone to occur, which are of sufficient intensity to promote spore germination; to facilitate matters, other anaerobes may intervene which are less exacting than *Cl. chauvoei* in the matter of reduced oxygen requirements and whose growth in

any case will be favoured by well-fed muscle. It may be that an explanation is to be sought on the lines of Carlström's work on equine myoglobinuria referred to above, *viz.*, damage to well-nourished muscle by lactic acid liberated in excess, though no evidence in support has been obtained here.

Mr. V. Rajagopalan has also suggested to me that a liberation of sulphydral groups may occur in muscle damaged by lactic acid and that this may effect the necessary lowering of oxygen tension. In the same connection it may be mentioned that muscle changes of obscure and unexplored nature, described as "myositis" and "degeneration of muscle," are known to occur in lambs first turned out to pasture in spring. Finally, it is noted (Howie and Cruickshank, 1947) that muscle extract itself may enhance the lethal effect of spores. This was so when fresh human muscle juice was mixed with anthrax spores and injected intraperitoneally in mice.

The hypotheses outlined are not exhaustive and it may be that the solution will not be found until there is a fuller understanding of the physiology and pathology of muscle under the different conditions of animal life.

SUMMARY

Experiments have shown that by raising the nutritional level of sheep by feeding a diet rich in protein, their susceptibility to black quarter spores, injected intramuscularly in "borderline" doses, is increased. Sheep in poor muscular condition either fail to become infected or the development of lesions is retarded. A single experiment planned to produce a similar effect by addition of molasses to the diet failed.

The reasons for these differences in susceptibility, or more explicitly differences in conditions which govern the germination of latent spores, are discussed.

The glycogen content of the muscles of the well-fed sheep is higher than that of the less well-fed sheep, the differences between the mean values in these experiments being highly significant. There was no difference in the mean values for muscle or blood lactic acid. It is probable that the higher glycogen level of the well-fed sheep is concerned not with spore germination but with multiplication of the bacilli after the spores have germinated.

Sheep, whose movements had been restricted for a few days, were given forced exertion immediately after intramuscular inoculation, but there was no good evidence that their susceptibility was modified by this treatment.

ACKNOWLEDGMENTS

The section of the work dealing with glycogen and lactic acid determinations was carried out by Dr. N. B. Das, of the Indian Veterinary Research Institute, Mukteswar. My thanks are also due to Dr. K. C. Sen and to Dr. M. K. Srinavasan for skilled assistance in the feeding and management of the sheep.

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PATHOGENESIS OF BLACK QUARTER

III. Effect of Chilling Agencies

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INTRODUCTION

The purpose of these experiments was to see if resistance of the body to black quarter spores could be modified by the physical agency of over-cooling. As pointed out in the first paper of this series (1948a), it is at least conceivable that the occurrence of black quarter in India at the time of the monsoon rains is connected in some way with an increase of host susceptibility caused by the wet conditions. Experimental work on this hypothesis would not be without interest in colder countries also. In a similar connection, Borthwick (1934) has shown that by exposing guinea-pigs to cold their susceptibility to *Cl. septicum* cultures by the mouth can be considerably increased.

At the time the present experiments were started, it was not realised that the bodily resistance of apparently healthy young sheep to implanted spores is so variable and that this variability is probably bound up with nutritional causes (Minett, 1948b). There are two types of experiment that can be used in this class of investigation, and both have been used in the present study. In the first, the same dose of spores—in an amount which from previous trials is unlikely alone to infect—is given to two or more apparently similar sheep and shortly after half of them are subjected to the supposed debilitating influence. In the second, the spores are given to one or more sheep and a delay of two days or so is allowed so as to decide whether black quarter directly due to the inoculation is likely to arise ; if symptoms do not appear, the supposed debilitating agency is then applied to all. To all sheep that do not respond, an injection of calcium chloride can be given later at the site of the previous inoculation to see if spores are still persisting. For reasons mentioned above, the second type of experiment is the more satisfactory for the present purpose. The first kind of experiment would involve the use of larger numbers of animals, and the results might require statistical treatment.

In this work, which extended over the years 1941 to 1947, the effect of local chilling and general chilling of the body has been tested. Heated spore suspensions, prepared at various times during this period and kept in cold store, have been used. Nearly all the sheep were yearlings ; both males and females had to be used but so far as could be judged this did not influence the results. Incidentally, it was of interest to note whether the treatment would have any other deleterious effect on the animals. A few experiments were

also done on the effect of local overheating, since it is stated that heating of an extremity (tail) can excite a black quarter oedema in this unusual situation. In cases where the results of experiments were negative, it will be sufficient to give a summary of the work done.

LOCAL CHILLING

Spores in total numbers stated were given subcutaneously, just above the fetlock usually of two limbs, or at various points in the back. In the former case, the sheep were made to stand to above the fetlocks in iced water or cold mud, and in the latter case ice packs were applied. Chilling, presumably, would have the effect of retarding the lymph flow and removal of the spores from the site of injection.

Fetlock.—The iced bath, at a temperature usually ranging from 4° C. to 8° C., was applied for two to six hours on each occasion. Excluding experiments in which some of the sheep developed black quarter, which may have been directly due to the inoculation, there remain eight sheep which showed no symptoms. Four of these were given spores estimated at (millions) 0·08, 0·08, 6·5 and 13·0; in the other four no proper estimation was made, but the number was probably at least 0·1 million. Four of the eight sheep were put in the bath immediately and again 24 hours (= day 1) after inoculation, two were bathed once, *viz.*, on days 3 and 7, and two were bathed twice, *viz.*, on days 3 and 10 and on days 13 and 18. It happened that two sheep died of peritonitis two and four days after their baths, *viz.*, the sheep bathed once on day 3 and the one bathed twice on days 3 and 10. Two uninoculated sheep subjected to the bath remained normal; incidentally, repeated blood cultures made on one of these showed no organisms. It was further noted that cooling of the extremities never caused more than a slight decline and, at times, an actual rise of rectal temperature.

Other sheep were held for five to ten hours in the day in a mud bath. Ice was added to the mud at intervals so that its temperature ranged from 8° C. to 12° C. Seven sheep exposed immediately after inoculation and again on day 1 did not develop black quarter. Three of these were given spores estimated at (thousands) 3·5, 3·5, 35·0; in the other four the number was probably at least 100,000. One sheep exposed for ten hours during the second day after inoculation (day 2) died of black quarter on day 3 with lesions in the subcutaneous tissues over the right thigh and loin. Of three uninoculated controls, subjected to the bath on two or three occasions, two remained normal and one died from unascertained cause. The iced mud usually caused a slight rise in the rectal temperature.

A few tests were made on the effect of heating of the extremities. Eleven sheep were inoculated in two fetlocks with spores and then made to stand up to the knee and hock in water maintained at about 45° C., for four to six hours at each exposure. This caused a rise

of rectal temperature. Seven of the animals were immersed immediately after inoculation and again on day 1; the rest were bathed on days 3 to 6 after inoculation. Two were given spores estimated at 70,000, three others 440,000; with the other six the number was probably at least 100,000. The results were negative.

Back.—Spores were injected at four points about the lumbar region and shoulder. Crushed ice enclosed in sacking was then bound over the skin at each site for two to six hours, causing some fall of rectal temperature. Five sheep were used for experiment, the pack being held in position on day 3 in one animal, on days 3, 4 and 5 in another, and on days 2, 3 and 4 in the other three. The last three were given spores estimated at 60, 600 and 6,000, the two former at (thousands) 88 and 880. None developed black quarter. In all cases injections of calcium chloride were given later at each spot. In two of the sheep, which had been given three ice-pack treatments, persisting spores then germinated and the sheep died of black quarter.

GENERAL CHILLING

Spores were injected in what was regarded as a "safe" dose, either intravenously or subcutaneously at three to six points on the back, or intramuscularly. In the latter case they were given either at one point at the back of the thigh or at four points about the thighs and shoulders. A few sheep were given spores by the mouth or by the nose. Immediately or after some delay, the sheep were exposed to the chilling influence which consisted either of an artificial shower or of natural rain. Rectal temperatures were noted at intervals. Often the shower was repeated, or with natural rain the animals were exposed on several consecutive days or through the night. Most of the animals had been clipped or reclipped a day before inoculation. The results of all the work falling under this heading are summarised briefly in Table I, wherein the experiments are given serial numbers. In any one experiment the same suspension was used and at least one experimental sheep and its unchilled control received the same dilution. It therefore becomes unnecessary to go into exact details of the numbers of spores given and the results can be considered collectively. Where both the experimental and its fellow control reacted, the result is omitted.

Artificial Shower

This was applied for 30 to 60 minutes, and was heavy (up to 10 inches in the hour), since a rapid fall of body temperature to about 97° F. was desired. The sheep were then allowed to dry off. The water temperature varied from 60° to 70° F. and the air of the drying shed usually 70° to 80° F. Nearly all the experiments were done at Izatnagar, between January and April, though not all in the same year.

Spores Intravenously, by Mouth or Nose (*Experiment* 1).—Five sheep were given spores intravenously in numbers estimated at (thousands)

TABLE I
INOCULATIONS IN SHEEP SUBJECTED TO GENERAL CHILLING

Experiment	*Inoculation*	*No. of sheep inoculated*	*Results*
		Artificial Shower	
1	Intravenous	Experimental 5	Negative
	Mouth	„ 2	„
	Nose	„ 2	„
2	Subcutaneous Three points (back)	„ 8	„
	Intramuscular		
3	One point	„ 2	Negative
		Controls 2	1 positive, 1 negative
4	„	Experimental 12	1 „ 11 „
5	Four points	„ 9	8 „ 1 „
		Controls 9	2 „ 7 „
6	„	Experimental 14	3 „ 11 „
		Controls 14	1 „ 13 „
7	„	Experimental 2	Negative
		Controls 2	„
		Natural Rain	
8	Subcutaneous Six points (back)	Experimental 4	Negative
	Fetlock	„ 1	„
		Control 1	„
	Four points (back)	Experimental 2	1 negative, 1 peritonitis
		Controls 2	2 „
	Intramuscular		
9	One point	Experimental 6	1 positive, 5 negative
		Controls 5	Negative
10	„	Experimental 6	„
		Controls 6	„
11	Four points	Experimental 8	1 positive, 7 negative
		Controls 8	2 „ 6 „

Note.—The one suspension was used for Experiments 2 and 4, a second suspension for Experiments 7 and 11.

Summary of Intramuscular Experiments

Shower	Experimental	12 positive, 27 negative
	Controls ...	4 „ 23 „
Rain ...	Experimental	2 „ 18 „
	Controls ...	2 „ 17 „

15 and 37·5 in two cases and at probably at least half a million in the other three. Two hours later they were put under the shower and with three of them the shower was repeated once or twice. Two

sheep were given spore suspension by the mouth and two by the nose, in the latter case slowly through a rubber tube. The numbers of spores were unestimated but probably ran to several hundred thousands. Next day they were put under the shower. The body temperature fell satisfactorily in all these animals, but none developed black quarter.

Spores Subcutaneously. — Excluding cases where black quarter developed which may have been due directly to the inoculation, there were eight experimental animals which showed no response (Experiment 2). Each was given about 9,000 spores in all at three points on the back. Four of them were exposed immediately after inoculation and again on day 3, the others were exposed once only on day 3. In all these cases the body temperature was brought down by the shower to about 97° to 98° F.

Spores Intramuscularly.—There were 16 cases (Experiments 3 and 4) where sheep were injected with spores at one point only. In Experiment 3 the number of spores inoculated was estimated at 1,000 and 100. One of the control sheep, injected with 1,000 spores, developed black quarter two days after inoculation. The two experimental sheep were given a single shower immediately after inoculation. The 12 sheep in Experiment 4 had been given extra concentrates for a month ; four of them were given 7,500 spores and eight about 3,000 spores each. One was given a single shower immediately after inoculation, five were given the shower immediately and again on day 3, and six were given a single shower on day 3 only. The only sheep to react was the one getting the single immediate shower and it belonged to the group given the larger dose of spores.

With sheep inoculated with spores at four points in the musculature, there is naturally a greater chance of the disease developing. In Experiment 5, carried out at intervals between February 9th and March 3rd, 1942, there are nine cases where comparison can be made between exposed and unexposed sheep. The inoculated animals were given the shower immediately after inoculation. Of the nine sheep exposed to the shower, eight developed black quarter, while of nine controls only two got the disease. In these two cases the lesion was definitely less extensive and developed more slowly than in the corresponding experimental sheep. There are two points to be noted. On one occasion the two animals selected for the shower were in rather poorer condition than the two controls and thus were less likely to develop black quarter. Nevertheless, they got the disease. On another occasion the two controls were given ten times the number of spores injected into the two shower sheep. In spite of this, one of the shower sheep developed black quarter ; the other three escaped.

Experiments 6 and 7 were made on similar lines to Experiment 5, but all sheep had been given extra concentrate rations for one to three months before test. In Experiment 6 there were in total 14

experimental sheep and the same number of controls ; three of the former and one of the latter developed black quarter. Experiment 7 was quite negative.

Natural Rain

Most of the experiments were carried out at Mukteswar where, at the monsoon season, the rain water temperature is usually about 60° to 64° F. in the day time and rather below 60° at night (Minett, 1947). At times, when rain-storms threatened or had already started, the sheep were inoculated and immediately tied in the open, sometimes remaining out during the night. In most instances controls were inoculated and kept under shelter.

Spores Subcutaneously (*Experiment* 8 ; *July*, *August*, 1941).—Ten sheep were used, but black quarter failed to develop in any of the ten. Four of them each received spores, estimated to total 237,000, at six points on the back. They were then exposed to intermittent rain from the time of inoculation for four consecutive days. The total rainfall during the four days was 3·09 inches. During the first two nights they were out. All of them showed some fall in rectal temperature. Eight days later, calcium chloride was injected at the previous inoculation sites, and all developed black quarter.

The remaining six sheep in Experiment 8 were used as follows. Two were given spores above two fetlocks, total number 80,000 ; four were given spores at four points on the back, total number 160,000. One of the last four died of peritonitis on the ninth day. The intention was to expose three of the six sheep to rain, but as by the third day it had not rained water was poured over their bodies at intervals for two hours on this and the fourth day, while on the fifth day they were exposed to actual rain (34 cents fell in two hours in morning). The other three, which had hitherto acted as controls, were exposed to rain on the fifth and sixth days, being left out on the fifth night. On the fifth day, 82 cents fell on the sheep. On the 10th and 13th days after the spore inoculations, the five sheep then alive were injected with calcium chloride with the result that four of them developed black quarter.

Spores Intramuscularly.—In Experiments 9 and 10 the inoculations were made at one point only. In Experiment 9 there were six experimental sheep and five controls. In two experimental sheep and the corresponding controls an estimated dose of 35 spores was injected, in all other cases 3,500 spores. In the one positive experimental case the larger dose was used. The experiment was conducted in three parts, on July 2nd, 16th and 29th, 1942.

July 2nd.—Two experimental sheep, Nos. 36 and 37, were injected at 2.45 p.m. It rained from 3 to 4 p.m., and again heavily from 9.20 to 10 p.m., when they were brought into shelter. No. 37 was a small sheep in fairly good condition and it showed some fall of rectal temperature though it had

shivered well. No. 36 was a large sheep in fair condition which did not shiver and its temperature rose in spite of the rain. Next day (day 1) No. 37 had a black quarter swelling, while No. 36 remained well. On day 3 No. 36 was exposed at 8 a.m. to light, fickle rain lasting for two hours. Then, owing to lack of rain, water was poured over its body every 30 minutes for three hours. This caused some shivering and the rectal temperature did not fall. On day 17, No. 36 was again exposed to heavy rain which caused some temperature fall.

July 16th.—Two experimental sheep, Nos. 38 and 39, were first exposed at 9 a.m. on day 3 after infection. During this day and the following night it rained intermittently. On day 3, at 6 a.m., heavy rain began and No. 39 (a small female) had to be put in shelter after two hours since its temperature had fallen below 96° F. No. 38 (large male in good condition) remained out till 4 p.m. No. 39 was again exposed on day 5 to two hours' heavy rain. Neither sheep, nor the single control, became infected.

July 29th.—Experimental sheep, Nos. 40 and 41, were given the smaller dose (35) of spores at 8 p.m. They were then exposed for 24 hours ; for the first four hours of this there was strong drizzle rain and next day it rained intermittently. The rectal temperatures of these sheep fell but slightly and were usually rather higher than the controls. Both shivered well.

In Experiment 10 (August, 1942) there were six experimental and six control sheep, none of which developed black quarter. The number of spores injected in this experiment varied from 25 to 250,000.

August 15th.—Experimental sheep, Nos. 42 and 43, were injected at noon and immediately exposed for 24 hours, during which time there were three heavy storms lasting for six hours. The rectal temperatures fell to 99·6° F.

August 18th.—Experimental sheep, Nos. 44 and 45, were injected about 10 a.m. and at once exposed for 24 hours to rain, drizzling or intermittently heavy. No. 44 (small, thin female) died on day 6 but black quarter was not diagnosed. Its rectal temperature had fallen to 98·6° F. during the rain, while that of its companion (large female) had risen somewhat.

August 28th.—Experimental sheep, Nos. 46 and 47, were injected about 10 a.m. and at once exposed for 24 hours to conditions much like those on August 18th. No. 46 died on day 6 but black quarter was not diagnosed. Its rectal temperature throughout the exposure was consistently lower than that of No. 47 but did not fall below 99·2° F.

In Experiment 11 (July, 1947, at Mukteswar), the sheep were injected at four points in the musculature. Of eight experimental sheep, one was positive and, of eight controls, two were positive. One portion of the experiment requires notice.

On July 25th, four experimental sheep and a similar number of controls were injected about noon and immediately exposed for six and a half hours. The conditions were quite suitable for a satisfactory experiment since a rainfall of 1·83 in. was recorded at the station on the morning of the following day, and the bulk of this fell while the sheep were exposed. On day 2 they were again exposed for four hours to rainfall of nearly an inch. The rectal temperature of one sheep fell to 99·2° F. and the others to about 100° F. None of the eight sheep injected on July 25th developed black quarter.

DISCUSSION

There is little in these observations to show that chilling agencies are concerned in the pathogenesis of black quarter. Moderately severe local chilling did not induce germination of spores lying in the subcutaneous tissues. With regard to general chilling of the body, the only experiments that were at all suggestive were those in which a heavy shower was used, but in the face of so many negative results, and with so large a margin of chance in the selection of the sheep, it would undoubtedly be unwise to place reliance on cases that were positive. Still less is there any indication that natural rain has any importance, even when it causes a severe fall of body temperature. Monsoon rain at Mukteswar commonly occurs as heavy storms of relatively short duration, though at intervals one may expect rain which is more or less continuous for 18 hours or more and which is intermittently heavy. The water temperature, however, is usually little below 60° F. and clipped sheep in reasonable condition suffer little reduction of body temperature. Whether prolonged exposure to much colder rain would have a different effect cannot be said.

The final point is that nothing has come to light in these experiments to favour the hypothesis that spore germination is connected with the production of excessive amounts of lactic acid, though many of the sheep were in good condition.

SUMMARY

The influence of local and of general chilling and of local heating has been tested in relation to the pathogenesis of black quarter.

Spores in numbers non-infective by themselves were injected into sheep subcutaneously at the fetlock or over the back. Local chilling by standing the animals in iced water or mud, or by applying ice-packs to the back, failed to induce black quarter but the persistence of the spores could sometimes be demonstrated later by injecting calcium chloride. Sheep exposed to local chilling occasionally died of peritonitis. Local heating was without effect.

In testing the effect of general chilling, spores were inoculated intravenously, subcutaneously or intramuscularly, and the sheep were then exposed to heavy artificial showers or to natural rain. Although a number of positive results were obtained with the showers, it would be unwise to place much reliance on them. Natural rain under monsoon conditions at Mukteswar was not found to increase the susceptibility of the experimental animals.

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INDUSTRIAL FLUOROSIS OF FARM ANIMALS IN ENGLAND, ATTRIBUTABLE TO THE MANUFACTURE OF BRICKS, THE CALCINING OF IRONSTONE, AND TO ENAMELLING PROCESSES

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INTRODUCTION

In the summer of 1938 one of us (F. B.) was called in by veterinary practitioners to advise on the possible causation of a "mysterious lameness of cattle" near Stewartby, in Bedfordshire. Clinical examination and proximity to a large concentration of brick kilns suggested "industrial fluorosis" of the type described early in the century by Bartolucci (1912), in association with a superphosphate factory in Italy, and this diagnosis was quickly confirmed by pathological and chemical examination of the bones of animals slaughtered for the purpose. Enormous values for fluorine were found, 1·0 to 2·7 per cent. expressed on the bone ash, as compared with normal values of the order of 0·05 to 0·08 per cent. During life, affected animals were found to be excreting large amounts of fluorine in the urine, even up to 68 parts per million as compared with less than five parts per million in urine of cattle from other areas, and analysis of urine, hay and pasture grass, was adopted as a means of mapping the affected area.

Because of pending litigation, the extensive studies made in 1938 and 1939 were not published in full at the time, but a discussion was opened at a symposium on fluorosis at the Royal Society of Medicine and a brief summary published in the Proceedings (Blakemore *et al.*, 1941).

Early in 1946 two further outbreaks of industrial fluorosis were encountered, one in association with the open air calcining of ironstone in Lincolnshire, and the other in association with a colour and enamel factory in Staffordshire. The former has been briefly reported upon by Green (1946) without full protocols. The latter, identified by D. W. P. Bythell, and further investigated on the biochemical side by Green, has been briefly referred to in the paper by Boddie (1947), but detailed protocols have not yet been published.

In the interval, the problem of fluorosis in farm animals in association with aluminium factories in Scotland was investigated by Boddie (1945, 1947), and the present writers have been frequently consulted by the Ministry of Health, Ministry of Agriculture and others in relation to fluorine hazards associated with industrial

* Clinical and pathological studies undertaken by F. Blakemore and T. J. Bosworth. Biochemical work by H. H. Green.

enterprises. It is therefore felt that a more fully documented account of the English investigations is called for, and the present communication aims at supplying this.

MANUFACTURE OF BRICKS

In the district concerned which, because of the peculiar suitability of the local self-burning clay, now carries a very large number of kilns operated by several companies in close proximity, enquiry showed that the appearance of the disease coincided with recent expansion of the industry and multiplication of kilns over the previous decade. Some measure of clinical fluorosis had probably occurred for several years before and escaped correct diagnosis, but by 1938 many farms over a belt of about ten miles and for a distance of about one mile in the direction of the prevailing winds from any set of tall chimneys, were obviously affected, and sub-clinical fluorosis was suggested by elevated urinary output of fluorine and elevated fluorine values of pasture grass, up to even greater distances.

An attempt was made to ascertain the extent of contamination of pasture by analysing samples of hay and grass obtained from different farms in the locality, with results shown in Fig. 1.

From this map it will be noted that the values for areas close to kilns are high, figures of 51 p.p.m., 84 p.p.m., and 90 p.p.m. (expressed on the dry matter of grass or hay) being found within a mile of the kilns in the direction of the prevailing wind (north east), as compared with normal values of 2 p.p.m. for grass from other districts and grass growing on similar soil but outside the wind belt, or on the lee side of high ground. Areas to the south west of the factories were little affected, if at all.

When carrying out the survey it was found difficult to get consistent results even with different samples from the same farm, and it was apparent that the distribution of the contaminant fluorine was very irregular. Values for grass taken at random from a single field varied from 14 p.p.m. to 23 p.p.m. and purposeful selection of grass according to length and position gave even wider variations. Although this might be partly explained by variations in washing off of surface contamination by rain according to the nature of leaf and stem surfaces, the main effect is attributed to the arbitrary surface swirl of the wind-borne contaminant according to variations in soil contour and association of longer with shorter grasses. Filtration by long grass could protect shorter grass and a small hillock could protect the grass on the lee side. This effect was obvious to the naked eye in a wheat field growing close to one set of kilns and directly in the path of the prevailing wind. Ears of wheat on the same stem were obviously much more bleached on the side facing the factory, and shorter plants shielded by taller plants were less bleached. The bleaching itself was, presumably, due to the sulphur dioxide associated with fluorine compounds in the flue gases and was only noticeable on ripening crops very close to factories.

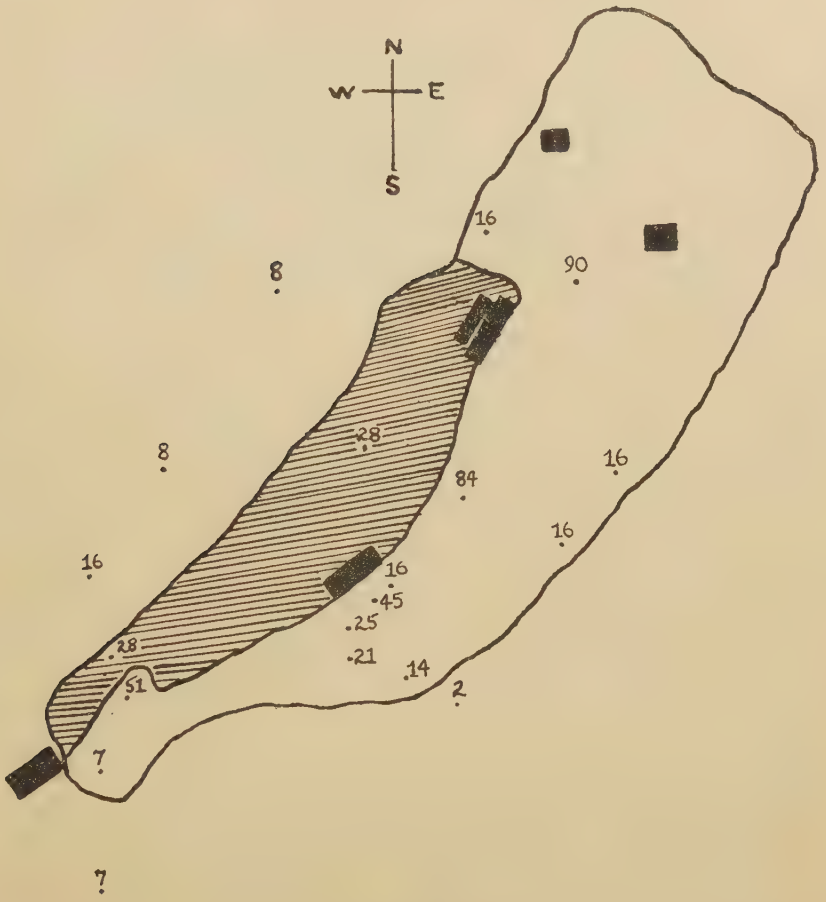

FIG. 1.—The distribution of fluorine on pastures in p.p.m. in relation to brick-making centres.

The lighter shaded area indicates the district in which fluorine intoxication of stock was reported and investigated. The pastural values in the darker area are also high, but no survey was made to ascertain the condition of the stock maintained there. The position of the kilns is indicated by the black squares. (Scale: $\frac{3}{8}$ inch to the mile).

In spite of variations of grass and hay contamination on any given farm, however, the general picture was clear and close correlation was possible between clinical symptoms in stock and average fluorine contamination of grass. In general, marked cases of the disease, as shown by lameness and pronounced skeletal changes, only occurred where pasture values exceeded 25 p.p.m. on a dry matter basis. Milder cases, however, evidenced by mottling of the teeth, were found on pastures of much lower content, and the extent of this area is represented in Fig. 1 by the farms showing values of 14 to 16 p.p.m. The whole of the clinically affected area com-

prised a belt about ten miles long by one mile broad, but sub-clinical cases would certainly have been picked out for some distance on each side of this belt if facilities for observation had been more extensive. The reason for the extension in length of the belt is indicated by the position of the aggregates of kilns of different brick companies, and although it was not possible to assess the damage done by each factory the broad generalisation may be made that from any relatively large collection of chimneys fluorine may be distributed to a distance of about a mile in the direction of the prevailing wind, in amounts sufficient to give rise to serious symptoms among cattle grazing on the pastures.

Where the aggregates of kilns are small, or the type of clay different, no clinical signs may be shown and no real interference with animal husbandry occurs. Urine samples from cattle in the vicinity of smaller brick factories in other areas have been examined on three occasions, but values did not exceed 8 p.p.m. and no clinical symptoms were reported in the animals concerned.

Urinary Values for Fluorine

Urinary excretion of fluorine by an animal is an obvious method of obtaining an indication of fluorine ingestion, and although the level at any given moment is influenced not only by the current rate of absorption from food but by the rate of excretion from the skeleton of fluorine stored in the past, it is invaluable in any studies of naturally occurring fluorosis.

The symptoms of advanced fluorosis in cattle are sufficiently characteristic for diagnosis, and when dental changes are present the disease is not likely to be confused with any other condition. Adult animals, however, when introduced on to affected farms, do not show dental changes and since minor osseous changes may be confused with other osteodystrophic disturbances it may be necessary to obtain further evidence of fluorosis during life. This situation may well arise, as for instance, on a farm in which dairy cows are bought in before calving but calves not reared *in situ*. In human fluorosis radiology has proved to be an important method of observing osteosclerotic changes, but in animals the method has not, for various reasons, proved very effective. In this investigation it was found that the most practical method of assessing the degree of fluorosis was to determine the current rate of fluorine excretion in samples of urine, easily obtained by catheter and rapidly analysed by appropriate technique. The modification of previously existent techniques used in this investigation, which enabled one operator to analyse 12 samples a day, is given in Appendix 2. This method of urinary diagnosis is not only useful in substantiating a clinical diagnosis since the clinician can get laboratory results returned within a day of receipt of samples, but is also invaluable in plotting the extent of areas of abnormal fluorine ingestion and the severity

of effects. Abnormal elevation of urinary values reflects incipient fluorosis long before any clinical signs appear.

In the investigations now recorded it was found that in advanced clinically detectable fluorosis urinary values varied from 16 p.p.m. to 68 p.p.m. with a preponderance of values well over 25 p.p.m., as compared with values for normal cattle of 2 p.p.m. at Weybridge and up to 6 p.p.m. for cattle grazing on fluorine rich soil at Cambridge. For animals on the edge of the affected belt, and sometimes showing signs of dental fluorosis, values of the order 11 p.p.m. were encountered. Young stock from other areas have been found with values as high as 7 p.p.m. but without dental fluorosis. It would, therefore, seem that a urinary level of 10 p.p.m. is somewhat critical from the diagnostic point of view.

In stating figures as parts per million of urine, as voided, comparisons are rendered difficult by wide variations in the concentration of the sample due to variations of water consumption. A better comparison is obtained by correcting all values to an arbitrary specific gravity, the figure 1·040 being taken in the present series. This correction is simply made by multiplying the observed fluorine value by the last two figures of the arbitrary standard S.G. and dividing by the last two figures of the observed S.G.

Normal Values.—With regard to normal values for fluorine, either in urine or in bone, a great deal of latitude must be allowed in interpretation of the word " normal." If clinical health and freedom from suspicion of fluorosis is taken as criterion, figures may be astonishingly high in comparison with values in the human subject. If " common values," as indicated by random urine samples and bones obtained from slaughter houses, are considered, the so-called " normals " become much lower although, particularly in the case of urine, they tend to be well above human values. The reason is not far to seek. Not only are the vegetative parts of plants consumed by grazing animals higher in fluorine than the endosperm of grain which constitutes so much of human food, but they are also liable to soil contamination. Although the fluorine compounds of soil are not readily available in digestion they are always present in relatively large amounts and doubtless contribute appreciably to daily absorption and excretion. This, in turn, influences quantities stored in the skeleton and may explain the wide variations given in the literature for normal bones of herbivora. Although there is room for much further work on the common normal range and on the rate of fluorine storage on experimental diets, our own provisional criteria are a common level of about 2 p.p.m. on urine for stall-fed animals in areas low in fluorine up to 7 p.p.m. for grazing cattle in areas high in fluorine, such as soils derived from the Oxford clay, without clinical symptoms until values exceed 10 p.p.m. For values on " bone ash " (the conventional mode of expression) fluorine values of 0·05 per cent. to 0·10 per cent. (500 to 1,000 p.p.m.) are

regarded as quite normal, but can be greatly exceeded before fluorosis is clinically detectable.

Excretion Rate of Fluorine of an Affected Case on Normal Diet

In order to estimate the rate of clinical recovery and excretion of fluorine after removal from the affected area, a cow was transported to Cambridge and there maintained on a normal ration for a period of eight months. Periodic samples of urine were taken and fragments of rib were removed surgically for analysis. The animal selected was not actually lame but was in poor condition and showed typical dental changes indicating exposure to fluorine from early life onwards, current exposure being indicated by the high value of 28 p.p.m. in the urine. Data are shown in the table.

TABLE I

EXCRETION OF FLUORINE FROM A FLUOROSED SKELETON

Time after abnormal fluorine intake ceased	*Fluorine values*	
	Urine of Sp. Gr. 1·04 p.p.m.	*Bone ash per cent.*
Initial	28	1·30
1 month	26	0·91
2 months	16	0·75
6 ,,	13	—
8 ,,	13	0·65

The animal rapidly improved on the Cambridge ration and was in excellent fat condition when slaughtered eight months after transfer. Although detailed histological studies were not made in this case, no gross skeletal lesions were observed at *post-mortem* in spite of a final fluorine value on the " bone ash " of about eight times the " common normal." Although it is possible that the urinary value had exceeded the 28 p.p.m. shown at the first sampling after transfer to Cambridge and may have been somewhat higher whilst the animal was still consuming fluorosed hay on the farm, the absence of material change over the first month is striking and the high value of 13 p.p.m. after eight months is illuminating. The normal value to be expected from the ration would be about 4 p.p.m. or only one-third of the final figure actually found. The fluorine content of the skeleton, as shown by the ash value for the rib fragments, fell materially during the first month, less rapidly during the second month and only very slowly over the ensuing six months. It is evident that replacement of fluorosed bone by normal bone is an exceedingly slow process, although the fairly rapid clinical improvement in moderately lame animals after removal from the source of ingestion suggests that those portions of the skeleton most subjected to stress and strain of weight and motion, and therefore most involved in rapid normal bone metabolism, are most rapidly rehabilitated; or, alternatively, that the most recently deposited fluorosed bone is soonest resorbed.

Rate of fixation of fluorine in bone, presumably as "fluorapatite" in place of the normal "carbapatite," does not seem to have been determined with precision, but observations of du Toit, Smuts and Malan (1937) indicate that actual retention in the bovine under conditions of fluoride feeding can at least reach 0·2 gramme per day.

The general picture, which is worthy of experimental confirmation, suggests that when abnormally high amounts of fluorine compounds are ingested a considerable proportion, depending upon availability in digestion, is absorbed, and that an immediate elevation of blood level occurs leading to an immediate elevation of urinary fluorine; that with continued ingestion the elevated blood level results in storage of fluorine in the skeleton, not by direct fixation but at a rate dependent upon the normal rate of bone metabolism, *i.e.*, as bone tissue is normally resorbed the fresh bone normally redeposited is laid down with fluorapatite instead of carbapatite as major mineral constituent; that excess of circulating fluorine is excreted but that a stage is finally reached when blood, bone, and urine are in equilibrium, and that on cessation of ingestion of fluorine the process is reversed; that urinary excretion is then maintained at a high level for long periods while fluorosed bone is slowly replaced by normal bone. Since the blood level also remains above the normal during the latter period, the resorption and apposition processes of normal bone metabolism are occurring in a *milieu* of elevated fluorine and the return of the skeleton to normal fluorine content is exceedingly slow.

At the time of making the original observations, the analytical technique did not permit of easy determination of the minute amounts of fluorine normally present in blood (of the order of 0·3 p.p.m.), and although this technique has now been further refined, exigencies of other work have prevented continuation of fluorine studies. But if one assumes a concentration of ultra-filtrable blood fluorine of about 100 times by the kidney, the increase in blood fluorine required to effect profound changes over a long period of time is exceedingly small. A change of urinary fluorine from 2 p.p.m. to 20 p.p.m. need only involve an elevation of blood level by about 0·2 p.p.m.

A picture of this sort explains many of the phenomena of naturally occurring fluorosis, notably the lack of correlation between pasture levels at any given moment (rapidly varying contamination) and the level of fluorine in the bones (a slowly varying change). In this connection it should be added that in the area investigated store cattle brought in for short periods (six months) rarely showed clinical symptoms, that badly affected animals always had a history of at least a year's residence on an affected farm and that clinical incidence seemed to be associated with dry periods and heavily contaminated herbage. For instance, in the dry summer of 1938 the grass on one farm showed 82 p.p.m., whereas the following wetter year it only

showed 36 p.p.m. This is attributed to more extensive growth, greater washing by rain and dispersal of factory gases over wider areas by higher winds. The increased incidence of lameness in the dry year, however, should perhaps be interpreted in the spirit of the "last straw which breaks the camel's back." A skeleton, after storing fluorine in previous years, may have reached a point of saturation at which it is unable to cope with the sudden influx of larger quantities. In the case of the cow recorded in Table I, the high level of 1·3 per cent. fluorine on the bone ash had been, as the dental changes show, gradually accumulated from calfhood onwards but the animal had not shown actual lameness. As indicated by the negligible change in urinary values during the first month after transfer, blood and skeleton were in a state of equilibrium at which a sudden increase of fluorine intake from higher pasture levels might well lead to disturbance of locomotion. Although fluorine values on bone ash of badly affected animals ranged from 1·3 per cent. to 2·7 per cent., the latter figure corresponding theoretically to an almost complete replacement of carbapatite bone by fluorapatite bone, there is no predetermined value at which lameness appears. It is believed that rapid skeletal changes in adult animals are more likely to induce lameness than slow changes, and lesions on articular surfaces have been encountered in another outbreak (page 286) associated with bone shaft values as low as 0·4 per cent. Dental changes in young animals can occur with still lower values for the bone ash of the whole tooth and of the skeleton.

Origin of the Fluorine in Affected Pastures

Although all the circumstantial evidence plainly suggested that the high fluorine content of the pastures was derived by aerial contamination from the neighbouring brickworks, it became necessary to prove that this was definitely the case, especially in view of possible litigation instituted by surrounding farmers. The possibility of water supplies being involved was ruled out by showing that local well water and the mains water of the district contained only 1 p.p.m. or less of fluorine. Examination of the bark of trees in the path of the prevailing wind immediately demonstrated the high level of aerial contamination. One such tree showed 146 p.p.m. on the dry matter of the outer bark scraped down to the underlying green bark, or 0·55 per cent. when expressed on the ash. The green bark below showed only 32 p.p.m. or about one-fifth as much. The outer leaves of cabbage in an adjoining field showed high and variable contamination, up to 50 p.p.m., but the protected inner heart leaves showed a normal value of less than 2 p.p.m. on the dry matter. Mangold leaves were contaminated but the roots, freed from adherent soil by skinning, also showed only normal traces. The soil upon which these vegetables were growing contained 200 to 380 p.p.m. but this is a common figure for the district and could in no way be attributed to accumulation of fluorine from the factories. Fluorine figures for grass on

fluorine rich soils in other parts of the country were 2 p.p.m. to 5 p.p.m., higher values than this being associated with obvious soil contamination.

To complete the evidence to the effect that high values in pasture were entirely due to surface contamination and were not contributed to by more readily available fluorine being washed into the soil and thence passed through the roots into the plants, a direct experiment was undertaken. Soil from Cambridge was transported to a farm near the brick works and two boxes, one containing Cambridge soil and the other local soil, were seeded with rye grass. At the same time soil from the affected farm was brought to Cambridge and rye grass again sown on it and on the local Cambridge soil. The grass growing in each box was sampled several times over the ensuing three months and showed average values, expressed as parts per million on the dry matter, as follows :—

Cambridge soil on affected farm	Cambridge soil in Cambridge	Local soil on affected farm	Local soil in Cambridge
110 p.p.m.	7 p.p.m.	88 p.p.m.	7 p.p.m.

Values for the soils themselves were 230 p.p.m. for the local and 110 p.p.m. for the Cambridge soil, the difference being due entirely to geological formation. The results clearly show that the amount of fluorine in the grass was not influenced by the amount present in the soil and that the high values on the affected farms were entirely due to local aerial contamination. It will be noted that the " normal values " of 7 p.p.m. at the Institute in Cambridge are somewhat high, in comparison with grass values found in other parts of Bedfordshire on soils of equally high fluorine content, but this may be due to slight soil contamination. Actual figures on individual samples varied erratically from 3 p.p.m. to 14 p.p.m., and an average on quite clean grass is just as likely to have been 4 p.p.m. as 7 p.p.m.

These data are in accordance with observations designed to ascertain whether phosphatic fertilisers containing fluorine affect the fluorine content of vegetation. For instance, Hart *et al.* (1934) found little variation in the fluorine content of the same crops grown on different plots of varying fluorine content, and quite recently experiments carried out by the Rowett Institute in Aberdeen (analyses conducted by one of us, H.H.G.) have shown that cereals grown with fluorine rich phosphate still contain only normal traces of the element. It would appear that fluorine compounds in small amounts, even if water-soluble, are rapidly fixed in insoluble form by the vast mass of soil through which plant roots range and merely add traces of the element to the relatively large amounts already present naturally.

Direct Proof of Association with Chimney Gases

To complete the picture, fluorine was actually demonstrated in the atmosphere of one of the worst affected farms, and by courtesy of the largest of the brick companies access was obtained to the works itself.

Atmospheric tests were made by installing an electric rotary pump on the lawn of the farm, switching on at times when the wind was blowing directly from adjacent chimney stacks and switching off when it was not. Air was drawn first through a small tube of cotton wool (1 cm. depth) and thence through tubes containing glass balls covered with dilute caustic soda. After a " total run " of 72 hours, the trapped fluorine was estimated and the machine calibrated to assess the total volume of air passed. The quantity found was of the order of 5 μg. F. per 100 cubic feet of air, a quantity very small in absolute measurement but sufficient to establish atmospheric contamination. Of this amount about half was trapped by the cotton wool as particulate matter, the other half being caught in gaseous form by the caustic soda. Most of the particulate matter was caught by the first few millimetres of cotton wool in the form of a dirty oily stain. Expression of fluorine in terms of the increase in weight of the cotton wool plug gave a value of 900 p.p.m. which, being much higher than that for the adjacent soil (230 p.p.m.), demonstrated that the particulate matter was not merely local atmospheric dust but definitely associated with the " oily mist " which could be seen drifting down from the top of the stacks in the gentle wind prevailing during the running periods of the rotary pump. With regard to the total fluorine, particulate and either gaseous or in such finely divided form as to escape the lightly plugged cotton wool, the value 5 μg. per 100 cubic feet actually found bears no relation to average aerial contamination since it was obtained under extreme conditions. Average values would be very much lower, perhaps of the order of 1 μg. per 1,000 cubic feet at the same point, and rapidly diminishing with distance from the chimneys. Nevertheless, in course of time such very small quantities would be quite sufficient to account for the relatively high values, up to 90 p.p.m., shown in Fig. 1 for grass and hay.

Flue gases within the chimneys themselves were then examined, using the same technique of the rotary pump in conjunction with the iron pipe used by the company itself for drawing off gas for control of the combustion processes. Practically no soot or dust particles were caught in the first tube containing cotton wool, but large amounts of an oily mist condensed in it to a mixture of aqueous and oily material containing about 50 p.p.m. of fluorine. The amount of condensation varied considerably in two consecutive runs, probably because of preliminary condensation in the sloping iron pipe in the stack itself, but the " apparent value " in terms of " gas sucked over " was about 1 μg. per litre or 30 mgm. per 1,000 cubic feet. The second tube, containing the caustic soda and catching fluorine

compounds not condensed in the mist or strained by the cotton wool, also showed about 1 μg. per litre of gas sucked over.

Since the quantities of fluorine in various forms passing up a single stack vary with the different stages of brick making and the number of kiln chambers it serves, a large number of tests would be required to obtain representative data, but the present impression is that the average amount emitted into the atmosphere is in the region of a few ounces per million cubic feet. This would represent a considerable annual tonnage from the great local concentration of stacks which developed over the 15 years preceding the enquiry.

Origin of the Fluorine

Samples of the clay used for brick making were found to contain 450 to 550 p.p.m. of fluorine (dry basis) and about 10 per cent. of organic matter, part of which could be destructively distilled in an oily form. The value of the clay is much improved by this organic matter, since after raising to ignition point with small quantities of coal it will continue to burn, thus decreasing the cost of manufacture. Comparison of baked and unbaked bricks indicated that about one-third of the total fluorine was lost in kilning, and from this an estimate could be made of fluorine emission in terms of the number of bricks produced over any given period of time.

In the process of brick burning a large number of chambers are served by a common stack, built very high and relying on natural draught for removal of vapours. These are arranged in such a way that the hot gases from chambers at higher temperatures are used to dry the wet bricks in the cooler chambers, and gradually raise them to ignition point, after which they finish themselves at bright red heat. Loss of fluorine obviously depends on temperature, surface of volatilisation, quantity of " combined water " in relation to " free water " and many other factors. To obtain some general information on the reactions, dry powdered clay was heated in an electric muffle for varying periods. Half an hour at 300° C. gave a darkish mass still containing some carbon, but from which oil had distilled off without loss of fluorine. This treatment followed by heating to about 500° C. for an hour gave a buff-coloured ash, from which most of the carbonaceous matter had been burnt off without, however, material loss of fluorine. The same treatment at 700° C. to 800° C. resulted in a small loss of fluorine, corresponding to reduction of the clay from 475 p.p.m. to 450 p.p.m. Ground clay gradually raised to bright red heat and held at about 1,000° C. for a further two hours gave a reddish coloured ash resembling that of finished brick with almost complete loss of fluorine—a fall from 475 p.p.m. to 65 p.p.m., or much greater than in natural brick burning. Although these simple laboratory experiments do not give a true version of the happenings in the kilns, where heating is effected in presence of circulating hot vapours including traces of sulphur acids from associated

coal, they do enable a general picture to be drawn of the whole process as it affects the livestock on neighbouring farms.

The origin of the fluorine is the self-burning clay rich in organic matter. As the temperature rises in the kilns water vapour is given off and at moderate temperatures a considerable amount of oil distils off. At still higher temperatures fluorine compounds, presumably silicon fluoride in the first instance, are liberated. Some brick dust is also swept off, although the infrequency with which the flues need cleaning and the comparative freedom of the flue gases from dirt at the sampling pipe, suggest that this is small in amount. Since the effluents from a large series of chambers at varying temperatures all pass up the same stack, they react and condense to give an aqueous oily mist together with gaseous products or particles so small that they are not easily filterable by cotton wool and are readily dispersed by wind over long distances. Both contain fluorine compounds but it is only the mist particles which are dense enough to be carried down on to the pastures within a mile of the stacks, where they adhere to the grass as a dust laden oily film and account for the ingestion of fluorine by grazing cattle. That the degree of contamination is dependent on the direction and speed of the prevailing wind can readily be observed by eye. On a still day the mist from the stacks seems to settle very close to the factories. A gentle wind distributes at a wider angle of drift and a high wind sweeps it rapidly away. The pattern of distribution of pasture contamination shown in Fig. 1 is thus very easily explained.

CLINICAL SYMPTOMS AND EFFECTS ON STOCK GRAZING IN THE DISTRICT

Detailed *post-mortem* examination was made on eleven bovines showing advanced clinical symptoms of fluorosis and specimen bones from several other animals were examined. The protocols of six representative cases, including fluorine distribution in various bones of the skeleton, are given in Appendix I.

General examination of the animals on the farms in the area revealed that the severity of the symptoms varied directly with the degree to which the herbage was contaminated, and the severely affected districts merged gradually into the normal areas. A great difference in the susceptibility of different farm animals was apparent, cattle being the most prone to develop symptoms. Signs of fluorosis were also found amongst wild animals, as evidenced by the fact that the femur of a rabbit caught on a severely affected farm contained 0·90 per cent. of fluorine on the " ash," a figure greatly in excess of a normal figure of 0·08 per cent. No gross changes were, however, observed in the skeleton, so that the absorbed fluorine evidently had little effect on the essential physiology of the bones. The features shown by different animals on severely affected farms were as follows.

Cattle.—The symptoms resembled those described by Hupka and Götze (1931) and others who have encountered fluorosis in the vicinity of industrial concerns. Evidence regarding the specific effect of chronic fluorine intoxication on the general health of the cattle was difficult to assess, but the growth of young stock was stunted, which is in agreement with the evidence of Roholm (1937) and others following the experimental feeding of sodium fluoride. In the advanced condition the appearance of the animals was striking. There was marked cachexia and the gait was stiff and laborious. The animals remained recumbent for long periods, and when called upon to rise often remained for some time on their knees as though afraid to complete the act. When standing they adopted a " wooden " stance, and stood with arched backs. Appetite was retained but the food consumption was reduced when the animals were at pasture owing to their inability to move about. A fall in the milk yield was reported by some of the farmers, but this may have been due to the reduced food intake since even advanced cases gave an apparently normal amount of milk when they were stall fed. Diuresis and excessive thirst, which have frequently been recorded as symptoms of fluorosis, were not observed and there was no diarrhoea which could be attributed to intoxication.

The lameness was usually associated with marked skeletal abnormalities. Enlargement of the sternum and inferior maxilla was often seen, and there was invariably an increase in diameter of the metacarpals, metatarsals and phalanges. Well defined exostoses were frequently present on the bones and they often extended over a considerable area of the diaphysis. Localised swellings were found along the course of the ribs, and an examination of the thorax for the presence of such protuberances was a valuable aid to diagnosis. Palpation of the bones caused considerable pain evidenced by struggling and shaking of the feet, but there was no evidence of arthritis.

Signs of dental fluorosis were evident in cattle which had been reared on the affected farms. The changes were restricted almost without exception to the permanent teeth, but in isolated cases slight mottling of the deciduous teeth was also observed. There was loss of lustre, pitting, and the deposition of a dark pigmentary substance in parts of the defective enamel (Fig. 2). In many cases the incisor teeth showed signs of excessive and irregular wear and the molars were sometimes similarly affected with a corresponding increase in the size of the teeth in the opposite jaw. The gums were normal, with the exception of one animal which showed severe gingivitis.

Despite the interference with calcium metabolism, which takes place most obviously in the skeleton, the incidence of milk fever and allied diseases in affected herds was low. Evidence that the blood calcium level was maintained even in severe cases was obtained by estimations on the blood serum of seven cows which all gave normal values of 10 to 11 mgm. per cent. The serum phosphate,

FIG. 2.—Incisor teeth (Case 5), showing mottling and erosion of the central permanent incisors. Enamel with signs of uneven wear.

as determined on two advanced cases, was also normal, being 6·7 mgm. per cent. in each. The phosphatase content in Bodansky units on the same two samples was 4·8 in one and 10·3 in the other, as compared with a control figure of 1·7. The blood picture is, therefore, similar to osteitis deformans (Paget's disease) in man, and suggests that the condition was not due to defective absorption but to metabolic disturbance in the bones.

The course of the disease in cattle was usually prolonged. Advanced cases were emaciated and unable to rise. The majority were then slaughtered as uneconomic animals, but it was found that if they were allowed to live with some attention to diet their condition improved after a recumbent period of several weeks. This was probably due to the fact that they were then hand fed with concentrates and the fluorine intake so reduced. They quickly relapsed, however, when turned out again on to contaminated pastures and permanent recovery did not occur naturally if the disease was clinically advanced.

In view of the possible danger to human beings consuming the milk of severely affected cows, a number of milk samples were analysed. It was found, however, that the fluorine values were not high, being less than 1 p.p.m.

Sheep.—The examination of sheep reared in the district revealed characteristic dental lesions in a high percentage of cases and such changes were common even in flocks kept on farms where the fluorine content of the grass was as low as 16 p.p.m. No general disturbance

in health was, however, apparent, nor were skeletal abnormalities or lameness observed.

In a flock which was kept on a farm known to be severely affected, many of the ewes gave birth to still-born lambs and the losses were attributed by the owner to the effect of fluorine intoxication. An examination of several carcases failed to reveal any bacteria of pathological significance and the blood of the ewes did not contain agglutinins to either *Vibrio foetus* or *Salmonella abortus ovis*. An analysis of bone material from one still-born lamb was made and the fluorine content of ash of a femur was found to be 0·10 per cent. While it has been reported by Evans *et al.* (1938) that small quantities of fluorine are present in foetal bones, the amount detected was considerably in excess of their reported values and of our own figure of 0·01 per cent. for still-born lambs from other districts. It would seem that fluorine readily passes into the foetal circulation. This is in accordance with the findings of Gaud *et al.* (1934), but its effect on the development of the tissues and the significance of chronic fluorine intoxication as a cause of abortion remain to be ascertained.

Horses.—On none of the farms was there any history of disease amongst horses which could have been attributed to fluorine. This apparent insusceptibility of horses was surprising as several animals were grazed on land known to be heavily contaminated and equines are naturally prone to osteodystrophic disturbances. A possible explanation is feeding on a high proportion of imported concentrates relatively to hay or to periods at pasture. Very few horses are bred in the district and we only had the opportunity of examining the incisor teeth of one which had been reared there. This animal showed typical mottling of the teeth but there was no history of lameness and an examination of the limbs failed to reveal any suggestive changes.

Poultry.—One farmer reported severe losses amongst his poultry, but on investigation it was found that neurolymphomatosis was responsible. Skeletal changes were not observed, but it was shown that the femurs of three specimens contained relatively high fluorine contents of 0·67, 0·27, and 0·40 per cent. respectively, whereas the figure for a normal control bone was only 0·08 per cent., expressed on the ash.

HISTOLOGICAL EXAMINATION OF BONES

The skeletal changes which have been recorded in cattle, sheep and pigs suffering from the chronic effects of ingestion of fluorine are summarised by Peirce (1939) as atrophy of the spongiosa, defective and irregular calcification of the newly formed osseous tissue and active periosteal bone formation which is considered to resemble the histological picture of osteomalacia. Changes having the same general pattern as these were observed in the disease described in this paper, but they varied greatly in intensity from case to case

according to the severity of the symptoms shown. No evidence of osteosclerosis was observed in any of the affected cattle. The condition is, however, quite a prominent feature of fluorine intoxication in a proportion of human cases, and it has been set up experimentally in certain other species of animals (Roholm, 1937). A case in an experimental bull has been reported recently by Pande (1944), and it seems, therefore, that it may occur naturally in cattle if favourable conditions obtain.

The histological changes seen in the skeletons of mild cases of bovine fluorosis in the Stewartby area were increased vascularity of the bone and periosteum, thinning of the trabeculae of the spongiosa and other early signs of atrophy. These included incipient porosis of the compacta (see Fig. 5), as well as irregularity in the staining reactions of the bony tissue. The appearances at this stage were similar in many respects to those resulting from the physiological withdrawal of calcium salts from the bones as seen especially in lactating animals. In early cases, therefore, it is difficult to be certain that the changes observed are pathological, but they tend to become greatly accentuated where fluorine intake has been high for any length of time ; there can then be no doubt as to their origin. The extreme fragility of the bones, and particularly the ribs, which characterises advanced cases is the result of extreme resorption (see Figs. 6 and 7). In sections of the ribs the compacta was seen to be reduced to a mere shell of porous bone which in places did not exceed one-twentieth of the normal width. The spongiosa was much atrophied and reduced to a network of wide irregular spaces as the result of general but uneven thinning of the trabeculae and the complete disappearance of some.

By this time attempts at regeneration of the bony tissue, which had appeared quite active in less severe cases, were largely in abeyance except at the site of fracture or of the formation of periosteal deposits ; elsewhere the process was restricted to the development of quite narrow seams of osteoid tissue with a few flattened cells present here and there on the surface. The staining of the trabeculae suggested uneven calcification and in places there were numbers of fine fairly uniform granules staining darkly with haematoxylin. This granular deposition of calcium salts was also found in other parts of the skeleton and appeared to be similar to that described by Sutro (1935), Ohnell *et al.* (1936), and Roholm (1937). The bone marrow was in part composed of haemopoietic tissue relatively poor in cells, as shown in Fig. 6, and in part represented by a loose fibrillar network in which numerous distended capillaries and in some cases extravasated fluid or blood were present. The periosteum was considerably thickened and hyperaemic at the site of exostosis ; it showed increased cellular activity leading to the production of collagen and numerous irregular trabeculae of osteoid tissue which became only very imperfectly calcified.

Examination of badly affected long bones of the limbs showed

eccentric atrophy with marked porosis of pre-existing bone and the formation of irregular periosteal deposits (Fig. 8). The internal lamellae were in part eroded and those that remained were of uneven width and very porous. The zone of Haversian systems was riddled throughout with spaces of varying size which were somewhat irregularly distributed. The enlarged Haversian canals were lined by wide seams of osteoid tissue (Fig. 9), and their walls were otherwise composed of partially calcified lamellae which varied considerably in thickness and staining capacity and as to number and evenness of distribution of the bone corpuscles. Evidence of lacunar erosion within the zone was by no means a prominent feature, and it appeared that breakdown of the compact tissue was largely brought about by vascular resorption. In exceptional cases, however, very active osteoclastic removal of bone was in evidence (Fig. 10), but as a rule this process was mainly confined to the exostoses. The surface layers of the bone were for the most part occupied by periosteal deposits, which were either directly applied to the zone of Haversian systems or separated from it by the remains of more normal-looking periosteal lamellae. The deposits were made up of loosely arranged trabeculae which often showed a concentric and sometimes a radial disposition. The various stages in their formation from the highly cellular thickened periosteum were apparent and the result was the development of very imperfect bone, irregular in structure and poorly calcified. Its rapid and widespread production throughout the skeleton was a marked feature of the advanced disease, but hardly less striking was an evident tendency towards its speedy resorption (Fig. 11). Bone formation at the surface and its breakdown by intensive lacunar erosion in the subjacent layers could often be found in adjacent fields under the microscope.

FLUOROSIS IN ASSOCIATION WITH THE CALCINING OF IRONSTONE

In some areas calcining of ironstone is carried out in the open air to dehydrate the fresh ore and decompose carbonate, so reducing the transport charges to distant smelting works. The area concerned, already briefly referred to by Green (1946), was in Lincolnshire. The industry here is of considerable antiquity, but the workings were considerably extended after 1939 because of war-time requirements. Reports of ill-health of cattle and considerable losses over ensuing years were received by the local veterinary practitioner from a farm adjoining a calcining area, and were brought specifically to our attention early in 1946 by G. A. Moore, of the veterinary field staff of the Ministry of Agriculture. Identification of the disease as typical fluorosis was promptly made on four samples of urine sent by him at our request. These showed values of 26, 28, 69 and 28 p.p.m. respectively which, together with the clinical description of lameness and cachexia, left no doubt that the cases were fluorosis of the type previously encountered in connection with brick making.

A visit to the area and *post-mortem* examinations on two bovines confirmed the diagnosis. Clinical symptoms and pathology were very similar to that already described and repetition of protocols is therefore unnecessary. Fluorine values on bone ash and from eight bone specimens from a badly affected heifer, ranged from 1·05 per cent. for rib to 1·55 per cent. for femur and sternum. The whole skeleton, therefore, was obviously fluorosed.

The local process of calcining consists in mixing the damp ironstone with about 7 per cent. of its weight of coal, and heaping the mixture into a continuous ridge running in circular fashion around the fringe of the area from which the ironstone is being lifted. The mixture is then fired at one point and ignition creeps along, the ridge being built up by addition of further coal and ore to a height of about 40 feet. The whole calcining process takes about six weeks for a typical ridge. The discharge of smoke is, therefore, only concentrated at one point for a relatively short period, but during that time contamination of adjoining pasture may be very heavy indeed and persist indefinitely in harvested hay crops.

Grass samples taken within a few hundred yards of a smouldering mound showed 2,200 p.p.m. fluorine on the dry matter, and the exterior of a straw stack on the affected farm, about half a mile from the ridge, gave a figure of 490 p.p.m. Straw from the interior of the same stack, taken by plunging the arm in about 2 feet, showed 70 p.p.m., the decrease being due to the filtration effect of the outer zone. The exterior of a more remote stack showed 280 p.p.m. Water from the cattle drinking trough contained only 0·5 p.p.m., so that the fluorosis manifested by the cattle could be entirely attributed to contamination of the vegetation, which was obviously very great when studied during the actual period of calcining. The farm in question, however, was wholly situated within one mile of the calcining area, and since very few animals were kept on adjoining properties it was not feasible to carry out a survey along the lines of that previously made in the brick-making district. The general impression was gained that damage to stock would be small except in the immediate vicinity of active calcining operations.

Origin of the Fluorine

Raw ironstone was found to contain about 0·12 per cent. of fluorine and the calcined product only 0·03 per cent., so that 75 per cent. of the total vanished during burning. The coal and coal slack contained 0·010 to 0·016 per cent. (100 to 160 p.p.m.) of fluorine and doubtless contributed to the damage. The desirable changes in calcinating are represented mainly by a fall in water content to about 2 per cent. and a rise in iron oxide (expressed as Fe_2O_3) from 50 to 70 per cent.

Observations on the temperature in the burning ridges, made at our request by the research department at the ironworks, showed that by the time the fire had extended half-way up a ridge of 40-feet

base and 40-feet height interior temperatures 2 feet from the outside were 1,100° C. on the fire line itself, 600° C. at 3 feet below this and 120° C. at 3 feet from the top. It is quite apparent that, despite the quiet appearance outside the ridge, vigorous combustion proceeds inside and that by the time the fire reaches the top the whole mass has been subjected to high temperatures. The proportion of fuel used is much higher than in brick burning and liberation of fluorine compounds may also be facilitated by the production of sulphur acids from the coal.

At any rate, fluorine compounds were condensed on the smoke particles and associated with them wherever they were deposited. It will be noted that in this case blackish smoke provided the heavier particles which carried the fluorine on to the pastures, whereas in the case of the brickworks the carrier was a whitish " oily mist." The dark cloud of smoke dragged at lower levels, apparently causing a more intense but less widely dispersed contamination.

FLUOROSIS IN ASSOCIATION WITH A COLOUR AND ENAMEL FACTORY

Emission of fluorine compounds from glazing processes using cryolite as flux and the use of hydrofluoric acid for etching purposes are recognised industrial hazards, but massive contamination of surroundings in secondary industries does not seem to be frequently reported. Early in 1946, however, our attention was drawn by D. W. P. Bythell, veterinary investigation officer stationed at Harper Adams Agricultural College, to an outbreak of fluorosis in cattle on a farm adjoining a colour and enamelling factory in Staffordshire. Urine samples of six cows showing clinical symptoms immediately confirmed his diagnosis, revealing fluorine values (corrected to comparable sp. gr.) from 19 p.p.m. to 37 p.p.m. Values on bone ash obtained later revealed figures up to 0·80 per cent. or ten times the normal.

Pasture samples taken from fields in the direction of the prevailing wind, again north-east, as in the case of the brick factories in Bedfordshire, revealed 776 p.p.m. of fluorine (on the dry matter) at a point 300 yards from the factory and 355 p.p.m. at 600 yards. Values at similar distances in a neighbouring field less directly contaminated showed 190 p.p.m. and 150 p.p.m. illustrating, as in the case of the brickworks, the variability of contamination according to countour of the land and swirl of the wind. Water supplies, both from pipe lines and from stream showed only 0·1 p.p.m. to 0·5 p.p.m. of fluorine. Clinical symptoms were not reported on more distant farms and no " mapping by veterinary catheter " was undertaken, but indirect evidence suggested that if subclinical cases had been included the affected belt would have fallen within less than a mile in the direction of the prevailing wind.

Clinical symptoms were not so severe as in the case of the brickworks and ironstone dumps, but after several years' exposure molar

teeth were worn down to the gums and permanent incisors were distorted and misshapen. The absence of pronounced skeletal lesions is attributed to the fact that during the winter the cattle concerned were stall fed and that hay purchased from another area was largely used, so that high exposure to fluorine was mainly confined to the summer periods at grass.

At a later date, F. A. Davidson, Divisional Veterinary Inspector of the Ministry of Agriculture, sent bones of an animal dealt with under the Tuberculosis Order in the same area. No pronounced clinical symptoms had been reported, but although molar teeth appeared sound one of the permanent incisors showed black markings. The ash of this tooth showed 0·34 per cent. of fluorine, and that of a metacarpal bone of the same animal showed 0·38 per cent. or about five times the normal value. Lesions on the articular surfaces of the bone were observed at *post-mortem* and subsequent enquiry revealed occasional prior attacks of lameness.

Exigencies of other work prevented further study of this outbreak and of the type of fluorine emission from the factory, but the occurrence emphasises the need for closer consideration of industrial fluorine hazards.

NOTE ON DAMAGE TO VEGETATION IN ASSOCIATION WITH FLUOROSIS OF ANIMALS

In general, clinical symptoms in animals in the outbreaks discussed were not associated with pronounced damage to foliage, which, if it occurred at all, seemed to be confined to vegetation very close to the factories and then to be caused by products in the smoke or fume other than fluorine compounds themselves. In this sense damage would come under the general heading of " atmospheric pollution," but it should be noted that specific damage by fluorine has not been adequately studied. Damage to vegetation in the vicinities studied from the animal side by Boddie (1945, 1947) in Scotland has been reported by others, and in this connection it is perhaps worth reporting a few analyses carried out on fir trees by one of us (H.H.G.) on samples sent by a local forestry consultant. A sample of Douglas fir growing over a mile in the direction of the wind from a large aluminium factory at Fort William, showed fluorine values (on a dry matter basis) of 685 p.p.m. and 230 p.p.m. on the foliage needles, as compared with a value of only 10 p.p.m. for a similar fir from an unaffected area. Outer bark of branches and exposed roots showed figures of 28 p.p.m. to 70 p.p.m. as compared with only 1 p.p.m. for inner wood, clearly showing the surface nature of the contamination and the absence of transmission *via* the soil through the deeper roots. The most superficial layers of soil adhering to surface roots were also contaminated, but it is presumed that deeper layers would not vary much from natural figures. The forestry consultant himself confined his report to damage done by " fumes " emitted by the factory over the preceding ten years, but

more detailed study of the components of the fumes would seem desirable since the presence of fluorine on the vegetation is not proof of specific damage by that element. Damage to plantations was reflected by slight discoloration and defoliation of more susceptible species up to a distance of seven miles ; pronounced discoloration, defoliation, and death within one mile and even up to two miles.

FLUORINE HAZARDS WITH SPECIAL REFERENCE TO SOCIAL CONSEQUENCES

Apart from danger to farm animals and dislocation of agricultural economy arising from extension of industrial processes, the hazards to human health have to be taken into consideration and the whole problem of fluorine contamination may have to be reviewed in relation to town planning schemes of the future. A few points of contact between animal and human health, arising from our own investigations, may therefore be referred to.

Hazards to Human Subjects.—At least 28 occupations have been listed (Sappington, 1943) as providing possible fluorine hazards, and in one industry alone, the manufacture of superphosphate, over 4,400 tons of fluorine are emptied into the atmosphere of the British Isles annually (Gill, 1946). Since various coals contain anything from mere traces up to 175 p.p.m., any form of smoke contains some fluorine, and Crossley (1944) considers that anything over 85 p.p.m. in coal is a potential industrial hazard. In some areas of the country natural waters contain appreciable amounts of fluorine, the classical case being Maldon, Essex, where a figure of 3·5 p.p.m. for domestic supplies led to severe dental fluorosis (Ainsworth, 1933) which was associated (Kemp *et al.*, 1946) with developmental disturbances of ossification. Traces of the order of 0·5 p.p.m. are regarded not only as harmless but as beneficial in the sense of improving dental enamel and reducing incidence of dental caries, and it is not until figures exceed 1 p.p.m. that noticeable mottling and staining of teeth appears in children.

The hazard to industrial workers associated with the use of cryolite in the manufacture of aluminium in Scotland has been studied by Hunter (1946), an investigation parallel with that of Boddie (1947) on the animal side. The possible social consequences associated with our own work on industrial fluorosis have been considered by Wilson and Murray (1946), with whom we have been in personal contact and to whom we have supplied occasional advance data. These workers consider the repercussions on human beings worthy of further investigation.

In the course of our own observations on fluorosis of farm animals we have discussed matters with various public health officers and medical practitioners and made occasional fluorine determinations on urine samples of human subjects associated with farms and factories, but had formed the general impression that the hazard to

the human subject was negligible and that the problems of aerial contamination were entirely different in veterinary and human medicine. The explanation of course is very simple. With farm stock the source of danger is grass and hay and no ill effects have yet been attributed to inhalation. With the human subject on the farm the dietary risk is confined to exposed outer leaves of vegetables such as cabbage and lettuce, which are either discarded or washed, and in any case form such a small part of the total food intake that they can be ignored. Roots and tubers and cereal endosperm are unaffected. Within factories the human risk is presumably inhalation and ingestion of small quantities of fluorine laden dust, most pronounced in the case of workers with cryolite (Roholm, 1937).

A point of significance, however, in comparing hazardous levels of ingestion or inhalation in man and animals is the very different span of life. Against the scriptural " three score years and ten " of man must be set the English average " economic life " of seven years, comprising about four profitable lactations for the dairy cow. To this must be added the fact that minor skeletal changes or mottling of dental enamel are of no practical consequence as they do not affect mastication of food, milk yield, or weight at slaughter, and, as shown in earlier sections, extensive deposition of fluorine in bones can occur before serious consequences ensue. In the case of erect man, small changes over long periods of time can be significant and spinal deformity in later life can result from progressive degeneration of malformations commenced in youth.

In studying human fluorosis radiological studies have proved very useful and urinary output has been used as an indication of fluorine ingestion, but it is suggested that much wider use should be made of the latter technique in conducting surveys of industrial populations. Because of the simplicity of obtaining urine and the ease of conducting " mass analyses " on very large numbers of samples, statistical cross sections of any given population can readily be obtained. With regard to comparable urinary levels of practical significance, there appears to be great difference between human beings and farm animals, although only ruminants have been studied so far. As pointed out on page 271, fluorine values in grass and hay are higher than in human dietaries, and urine levels of cattle commonly range 2 p.p.m. to 6 p.p.m. (corrected to sp. gr. 1·04) according to locality, figures which would be regarded as alarming in the human subject. Even if difference in sp. gr. be allowed for and cattle figures based upon a human average of 1·02, the cattle normals would still range well above human normals and, from the absence of dental signs in young stock, it would appear that grass-eating animals are less susceptible to moderate increase of fluorine absorption. For man, McClure *et al.* (1944, 1945) have shown close correlation between fluorine in urine and in drinking water. Average urinary values for man on an ordinary city dietary and low fluorine water supply are about 0·5 p.p.m., and higher values tend to take

the level corresponding to the drinking water. Urinary values, unless corrected for variations in water excretion, are somewhat ambiguous, and specific gravity should, therefore, always be included when a figure is given. Although far from perfect, a correction to some arbitrary standard (say 1·02 for man and 1·03 or 1·04 for cattle) would seem the simplest way of obtaining comparable data in survey work. Since human urine can easily vary from 1·01 to 1·03, or even 1·04, in different human subjects and at different times, three-fold variations, in level expressed as p.p.m. without correction, can quite well occur merely as a result of recent consumption of fluid.

In survey work on both man and animals, it should also be remembered that the urinary level is conditioned by the current balance between blood and bones and high levels may be the result either of high storage of fluorine in the past or of current high ingestion. Hence, unless the rate of current ingestion can be determined by other means, urine samples in particular individuals should be taken at intervals (say quarterly) to clarify the picture. In the case of the values shown by the household of " Farmer X," supplied by one of us for the paper of Wilson and Murray (1946), the high figures (1·6 to 4·1 p.p.m.) might be accounted for either as temporary elevations caused by current ingestion of cabbages and lettuce heavily contaminated by recent smoke from the calcining ridges, or as " skeletal equilibrium values " corresponding to the hazard of many years. In the case of the Stewartby cow, discussed on page 272, the high value of 13 p.p.m. eight months after transfer to Cambridge could be very deceptive unless the whole history of the case were known.

Control of Industrial Contamination.—For existing factories constructed before fluorine hazards were understood, it would often be a very expensive process to make alterations sufficient to ensure freedom from atmospheric contamination and the methods adopted to control damage are dictated by economic considerations, but for new factories built under sanction of planning authorities special attention to design and insertion of " scrubbers " might well solve the problem. In the case of the brickworks referred to on page 268, the accident of intervention of the War Agricultural Executive Committee after 1939, and compulsory ploughing-up of grazing land, did much to mitigate the local damage to farm animals. As a permanent measure, it would probably be more economical for each company to buy up the agricultural land within a mile of its chimneys in the direction of the prevailing wind and turn the system of husbandry over to one compatible with the fluorine hazard —diminishing the area of grass by cultivation of potatoes and feeding roots, and substituting store cattle, of limited anticipated residence, for permanent dairy cows ; or, alternatively, to pay existing owners appropriate fixed compensation for modifying their agriculture to suit local brick making. So far as the human population in the affected

belt is concerned, there is as yet no evidence that the brick fume hazard is materially greater than the nuisance of general atmospheric pollution, or the numerous other hazards to which mankind is exposed.

Other industrial concerns, notably large aluminium factories, have the same problem to face and the issues are economic as well as scientific.

SUMMARY

An account is given of the occurrence in England of fluorosis in farm animals arising from industrial contamination of pasture in connection with the manufacture of bricks in Bedfordshire, the calcining of ironstone in Lincolnshire and the fumes from a colour and enamel factory in Staffordshire. It is shown that the contamination of vegetation is entirely superficial and not due to fluorine compounds carried down into the soil by rain. The origin of the aerial fluorine is discussed in relation to the industrial processes involved, and extent of damage related to distance from source and direction of prevailing wind.

For the high concentration of kilns in the brick-making area, recently expanded by several companies, a map showing levels of pasture contamination (7 p.p.m. to 90 p.p.m.) is given, and this is discussed in relation to the severity of clinical symptoms in animals. Considerable differences were observed in regard to susceptibility of different classes of stock. Representative clinical cases are recorded and the histology of skeletal changes is illustrated by microphotographs.

Urinary analysis was found to be a convenient way of diagnosing fluorosis, especially in sub-clinical cases where no dental signs or obvious skeletal changes were present, and the value of this simple method is emphasised for surveying areas of potential fluorine hazard in respect of either human or animal population.

By correlating urinary excretion with fluorine values on rib bone removed surgically in an animal taken away from the source of fluorine ingestion, it was found that about half of the original skeletal fluorine still remained after eight months and that, although marked clinical improvement occurred quite soon, urinary values remained far above normal (13 p.p.m.) in equilibrium with the very slow change of bone values in the later stages of skeletal reconstruction. A high urinary value may reflect either high current consumption of fluorine, or the skeletal storage of fluorine in the past, and close correlation in any given outbreak of disease between clinical symptoms, urinary output of fluorine and changing levels of pasture contamination can not be expected.

Points of difference between fluorosis in its veterinary and medical aspects are briefly discussed in relation to social consequences and the economics of control of fluorine hazards.

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Appendix I

Post-mortem findings

Case 1: *Ayrshire Cow, six years old* (*weight* 8 *cwt.* 10 *lb.*)

Symptoms.—Emaciation; appetite good; no diarrhoea; short cough suggestive of tuberculosis. Tendency to remain recumbent. Painful lameness accentuated by manipulating joints. Beading of several costochondral junctions and localised thickening in upper third of ribs. Evidence of periosteal irregularity of metacarpals. Enlargement of many bones, particularly the sternum, inferior maxilla, metacarpals and digits. The teeth were normal.

Measurements of Different Parts of Limb in Inches

	Affected animal	*Healthy animal of same age and beeed*
Coronet	14·25	10·75
Fetlock joint	10·75	8·75
Middle cannon	7·75	6·5
Lower one-third cannon	10·25	7·75

Post mortem.—All abdominal organs were normal except the kidneys which showed numerous white infarcts. The wall of the reticulum had been perforated and there was evidence of passage of a foreign body with consequent production of inflammatory fibrous tissue extending through diaphragm and along the floor of the thorax. The lungs and pericardium were not affected by this process. Several tuberculous lesions were present on the right parietal pleura, covering an area of approximately 3 sq. in. The mediastinal and bronchial lymphatic glands were tuberculous and a few caseous foci were present in the lung. There was marked thickening of the sternum; the ribs were fragile and it was almost impossible to dissect them from the vertebrae without fracture below the head. It was possible to break several ribs at a time through this site by manual manipulation. The bones had lost their normal lustre and were dull and chalky in appearance. The metacarpals and digits were much enlarged and the periosteum was irregular.

Case 2: *Cow.*—The animal was stated to have been on the farm for twelve months.

Symptoms.—It was in an advanced stage of the disease and remained recumbent for long periods, showing signs of extreme pain on rising. There was marked deformity of the thorax and localised swellings were present along the course of the ribs. The teeth were normal.

Post mortem.—When the carcase was hoisted for examination several ribs fractured, indicating their fragility. Tuberculous lesions were found in the bronchial, mediastinal and mesenteric glands. These lesions were hard and caseous. There was evidence of a foreign body having penetrated the reticulum but without causing abscess formation or involvement of the liver. The carcase lymphatics were normal and there was no widespread tuberculosis of serous membranes or viscera.

On the right side of the thorax the last five ribs showed pronounced exostoses at the junction of the middle and upper thirds, indicative of

previous fractures. The two anterior ribs were similarly affected in the upper third.

The changes on the left side of the thorax were not so marked, but massive exostoses were found along the course of some of the ribs. Periosteal thickenings were pronounced on the bones of the limbs below the tarsus and carpus (Fig. 3).

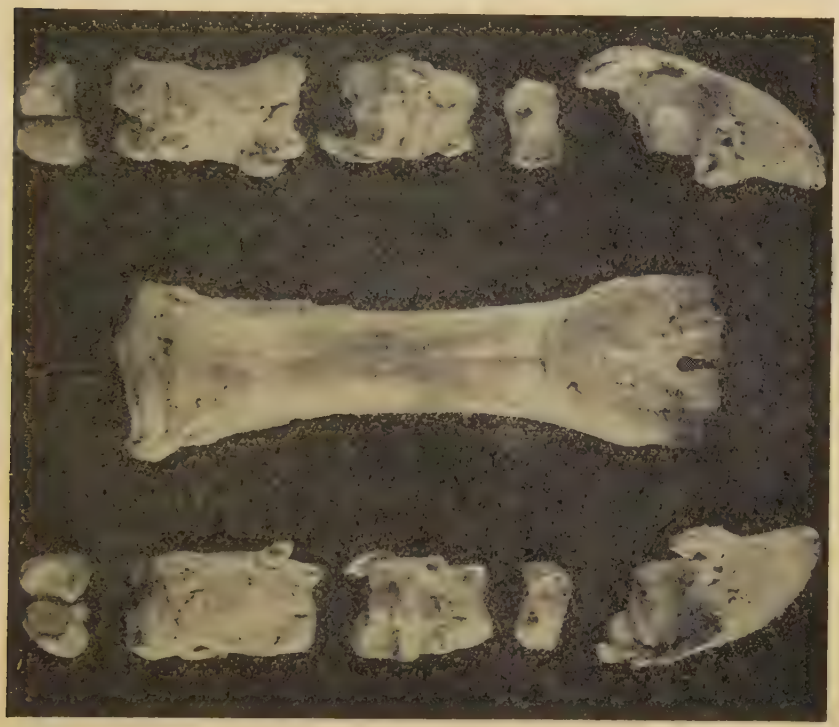

FIG. 3.—Bones from an advanced case of fluorosis showing widespread exostoses.

Case 3: *Cow, six years old.*—The animal had been in the owner's possession for two years.

Symptoms.—Emaciation and difficulty in walking. The claws were overgrown and there was evidence of laminitis. The teeth were normal.

Post mortem.—Penetration of a foreign body from the reticulum had caused localised fibrosis but no abscess formation or involvement of the liver. There was no evidence of tuberculosis. A tumour was present in the wall of the uterus. The abomasum was normal but the small intestine showed chronic thickening which diminished in degree towards the caecum. Examination of scrapings for acid-fast bacilli gave negative results.

Protuberances were found on seven ribs. It was impossible to dissect out the ribs completely owing to brittleness of the bones. The sternum was grossly thickened (see Fig. 4). Marked exostoses were present on the lower third of the metatarsals and similar changes were detected on the surface of many of the other bones.

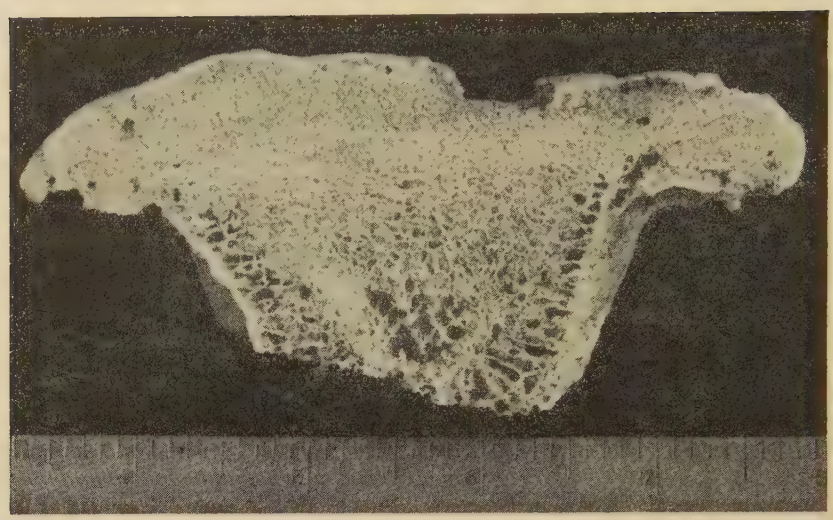

FIG. 4.—Cross section of the sternum near the ziphoid cartilage showing marked porosity. (Case 1).

Case 4: *Bullock*, 18 *months old.*—This animal had been unthrifty for some time and was examined to ascertain whether its emaciated state was due to fluorosis.

Symptoms.—The bullock was not lame but the incisor teeth showed evidence of fluorine intoxication.

Post mortem.—The liver was in an advanced state of cirrhosis and this condition was apparently responsible for its general condition. No changes were found in the other organs except those which could be traced to the state of the liver. The skeleton showed no signs of advanced fluorosis.

Case 5: *Black Poll Heifer, one and a half to two years old.*

The owner stated that the animal had shown no signs of chronic lameness although grazed on pasture on which others had become affected and it appeared quite healthy up to a few days before it was examined.

Symptoms.—It was observed to be rather unsteady in its gait and at times carried its head in a peculiar manner, holding it to one side or the other (more often the right) with the corresponding side of the face tilted slightly upwards. The permanent central incisor teeth showed pitted enamel and discoloration, but no other signs of fluorosis were detected clinically.

When examined immediately before slaughter the heifer could maintain its balance while moving in a straight line, but was apt to fall over with the slightest pull on the halter rope to one side or the other. When standing with its head in the normal position, however, it appeared a perfectly healthy young bovine in good condition.

Two possible explanations of the symptoms were considered; that ingestion of fluorine had produced a bone deformity resulting in pressure on the central nervous system and that the animal was affected with tuberculous meningitis. This was considered improbable because: (*a*) the animal had never shown excitement; (*b*) the owner stated that he had recently had one, if not two, other animals showing similar symptoms.

Post mortem.—On *post-mortem* examination widespread lesions of tuberculosis were evident. All the glands of the head were badly affected and the tonsils were also involved. In the chest the disease appeared to be confined to the bronchial and mediastinal glands which were extensively affected. There were extensive abdominal lesions affecting almost the whole of the parietal and visceral peritoneum, including that of the spleen and uterus. The capsule of the liver was greatly thickened, but not obviously tuberculous. The substance of the organ appeared normal, but the hepatic glands were tuberculous, as were the mesenteric and colic glands, almost the whole chain being involved and beset with caseo-calcareous lesions varying in size from a hazel nut to a pea. Likewise the inguinals and most of the peripheral glands, supramammary, popliteal and prescapular, were affected.

The bones were examined in detail but no gross changes suggestive of fluorosis were found except that the ribs were more brittle than normal as shown by the fact that when removed from the body it was possible to break them in the hands without using a fulcrum. The skull appeared to be normal except for the changes in the incisor teeth described above, and possibly a slight irregularity of wear of some of the molars. The wall of the cranial cavity was quite normal and there was no evidence of meningitis. The brain showed no gross changes. The internal ear and the tympanum including the *bulla ossea*, were filled with pinkish granulation tissue containing caseous centres, films from which contained numerous acid-fast bacilli. The condition was bilateral and there was little, if any, difference in the extent of the disease on the two sides. The Eustachian tubes were tuberculous, their walls were thickened, and the mucous membrane when scraped was very gritty. Smears of the scrapings showed acid-fast bacilli.

Case 6 : *Roan Shorthorn Heifer, two and a half years old.*

This animal was one of a group which had developed signs of fluorosis. It showed intermittent diarrhoea and was examined to ascertain whether this was a manifestation of fluorosis or was due to some other cause.

Symptoms.—Emaciation. Intermittent diarrhoea. Dental fluorosis. There were no prominent skeletal abnormalities or lameness.

Post mortem.—The most striking gross changes apart from general emaciation were in the intestine. The mucous membrane of the ileum was thickened and corrugated, and showed areas of congestion on the surface of the folds. The ileo-caecal valve was greyish-red in colour and dark red blotches were present here and there on the mucous membrane of the caecum. There was also an area about 2½ in. in diameter studded with small elevations each showing a hole in the centre as if due to some parasite. Smears from the ileum were rich in acid-fast bacilli, resembling *Mycobacterium johnei.*

The ribs were more brittle than normal and could be broken by hand after removal from the body.

In these six cases fluorosis was obviously complicated by other pathological conditions such as tuberculosis, Johne's disease and lesions arising from the penetration of foreign bodies. This made it difficult to form any definite conclusions regarding the effect of fluorine intoxication on tissues other than bones, but we were unable to find any consistent lesions, either by autopsy or histological examination, which would indicate that fluorosis had had any effect on the parenchymatous organs.

In advanced cases all parts of the skeleton were obviously affected and particularly the ribs, sternum, vertebrae, lower jaw and digits. The bones were more massive in appearance but of lower specific gravity than normal

and showed widespread irregularities of the surface when exostoses had occurred (Fig. 3). The periosteum was thickened and hyperaemic, the marrow centres enlarged and the marrow represented by a clear, gelatinous material having a reddish tinge. On cross section there was marked porosity of the bones, especially in the subperiosteal area (Fig. 4). The epithyseal lines appeared to be normal. In several instances the extreme degree of fragility of bone which may result from the condition was well illustrated (Case 1). After maceration the bones, instead of being smooth and glistening, were dull white and porous in appearance, and showed irregular, spongy, friable deposits on their surface. The fluorine values of various bones, expressed as percentage on the bone ash, of cases 1, 3, 4 and 6, together with two other affected cases, 7 and 9, and two normal animals, A and B, were as follows :—

Cow	*Sternum*	*Ribs*	*Phalanx*	*Inf. Maxilla*	*Humerus*
1	1·67	1·52	1·49	1·28	1·50
3	1·61	1·38	1·25	1·10	1·23
4	1·44	0·94	1·08	1·24	0·87
6	0·78	0·59	0·86	—	—
7	0·61	0·53	0·53	0·50	—
9	—	0·68	—	0·45	—
A	—	0·08	—	0·07	—
B	—	0·08	—	0·09	—

It will be noted that values for all affected bones are very high, ranging from about five to 20 times the normal, and that for any one skeleton the distribution of fluorine is fairly uniform.

Appendix II

Determination of Fluorine. Weybridge Modification, 1938

The modification is based upon the terminal procedure described by H. C. Lockwood, "Fluorine in Food Products" (1937), *Analyst*, **62,** pp. 775–783, and is designed for rapid determination of small quantities with simple compact equipment.

Principle.—Organic matter is destroyed by dry ashing in presence of magnesia, the fluorine separated by steam distillation as hydrofluosilicic acid at about 150° C. and estimated in the distillate by de Boer's fading reaction with zirconium alizarin lake.

Reagents required

1. Magnesium ashing mixture. An alkaline suspension, containing 1 per cent. magnesium oxide (A.R.), in 7 per cent. solution of magnesium acetate coloured with a drop of phenol phthalein. Shaken before use.

2. Approximately 4 N hydrochloric acid, prepared by diluting 400 ml. concentrated HCl to one litre. Analar HCl is sufficiently free from fluorine and chlorine for general use.

3. Approximately N. caustic soda. Analar.

4. Concentrated sulphuric acid freed from fluorine as far as possible. If the fluorine "blank" is too high for the purpose in view it may be reduced by diluting 450 ml. to 500 ml. with water and boiling in an open beaker back to approximately 450 ml.

5. 50 per cent. sulphuric acid. The acid above, diluted with an equal volume of water.

6. Broken glass. Glass tubing crushed to about 1 mm. in a mortar.

7. Dilute phenol phthalein. 0·01 per cent. in alcohol.

8. Sodium nitrite. 10 per cent. aqueous. Only required if free chlorine is suspected in the distillate or hydrochloric acid used.

9. Zirconium nitrate. 0·4 per cent. aqueous.

10. Sodium alizarin sulphonate. 1 gramme dissolved as far as possible in 100 ml. boiling absolute alcohol, filtered and then diluted to 250 ml. with absolute alcohol. Solubility is low but sufficient dissolves.

11. Zirconium alizarin "lake" used as final indicator. 6 ml. of solution 9 and 4 ml. of solution 10 diluted to 250 ml. with water. This pink reagent should be allowed to stand a few hours before use but has to be made up fresh every three days. After four days it becomes less sensitive and during the fading reaction with fluoride and hydrochloric acid the lake tends to precipitate in colloidal form.

12. Standard fluoride. A stock solution of $\frac{N}{19}$ sodium fluoride. Merck's "Natrium fluoridum purum" is suitable for direct weighing out and dissolves readily, but the standard is best checked by gravimetric precipitation as calcium fluoride in the usual way.

This stock solution is diluted 1 to 100 for use as standard, 1 ml. of the $\frac{N}{1,900}$ NaF being then equivalent to 10 μg. fluorine.

Apparatus

The complete equipment (with the exception of the water bath and muffle furnace) is shown in Fig. 12.

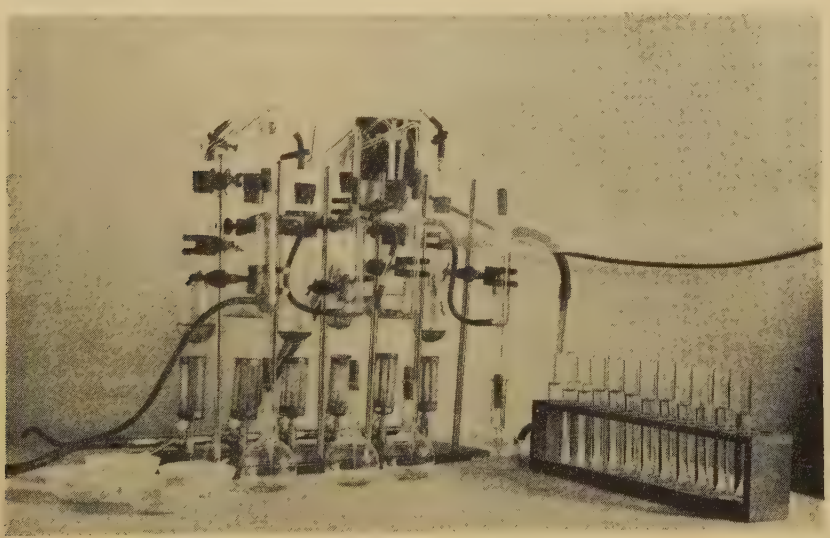

Fig. 12.—Apparatus useed for determination of fluorine.

The compact distillation stand is made by Gallenkamp & Co., London, and the glass parts blown by the " Scientific Glass-Blowing Co.", London.

The four distilling flasks are about 100 ml. capacity, of pyrex glass. Each is fitted with a three-hole rubber cork carrying a steam inlet, a thermometer reading to 150° C., and a distillation tube with simple " hole trap " providing a double channel for steam and so allowing condensing liquid to run back into the flask.

The two steam generating flasks, between the distilling flasks, are about 500 ml. capacity and are fitted with small tap funnels which can be used as safety valves or air inlets at the conclusion of distillation. Clips on the rubber connections serve to adjust steam into the two distilling flasks supplied by one steam generator. Adjustment of the micro-burners maintains any desired rate of steam passage.

The distillation tube slopes upwards so that a small amount of condensed water is continually returned to the flask, washing back any traces of acid spray entering the " hole trap." The small 5-in. condensers lead into collection flasks of 150 ml. capacity scratched on the neck to a mark representing 150 ml. of distillate.

The matching tubes shown in the tube rack are ordinary 15 cm. by 1·5 cm. rimless test-tubes, picked out as uniform in diameter so that when scratched with a 20 ml. mark the depth of liquid is approximately the same in all.

Procedure

The sample is weighed or measured into a silica basin, 8 cm. internal diameter being a useful size, 10 ml. magnesium ashing mixture added, and the whole evaporated to dryness on a steam bath. The quantity of sample to be taken is calculated from the " anticipated fluorine content " and although wide variations are permissible, a quantity containing about 100 μg. fluorine is the one which involves least subsequent manipulations of distillate. Since the final distillate volume is 150 ml. a convenient 15 ml. aliquot then contains 10 μg., a suitable quantity for subsequent colour matching. If more is used the distillate may have to be diluted. If less is used, it may have to be reduced in volume.

The dried residue in the basin is gently ignited over a low bunsen flame and combustion completed in a muffle furnace at a dull red heat, unnecessarily high temperatures being avoided. Occasional stirring with a glass rod expedites combustion, the magnesia facilitating rapid ashing as well as fixing fluorine.

The ash is scraped into the distilling flask and the main bulk washed in with about 5 ml. of water, 2 ml. 50 per cent. sulphuric acid are added to the basin, the residual ash detached with a rubber-tipped rod, dissolved in the cold and washed in with the minimum water (less than 10 ml.). Six ml. concentrated sulphuric acid are then added, to bring the final acid concentration in the distilling flask to the point at which it boils between 140° C. and 150° C. About 2 grammes of broken glass is added to provide silica and diminish bumping, the flask connected to the condenser and steam generator, and heating commenced. The contents of the flask rapidly rise to 140° to 150° C., any tendency to bump being controlled by an initial slow current of steam. Thereafter the micro-burners are adjusted so that the distilling temperature rises almost to 150° C. and the rate of steam provides about 150 ml. of distillate in the course of about an hour. All four distillations

can be run simultaneously, but they must be watched to ensure that no sulphuric acid spray passes the traps and reaches the distillates.

The receiving flasks are provided with 5 ml. N. NaOH and a drop of dilute phenol phthalein, to maintain alkaline reaction in the distillates despite varying quantities of chloride in the ash. Distillation is continued until the 150 ml. mark on the receiver is reached.

Fifteen ml. of distillate, *i.e.*, one-tenth of the whole, is transferred to a matching tube graduated at 20 ml. and approximately neutralised with 4 N hydrochloric acid (two or three drops sufficient). A further 2 ml. is then added to bring to appropriate final concentration of hydrochloric acid, and the whole mixed by inversion. Exactly 2 ml. of the zirconium alizarin lake is finally added (conveniently from a burette in routine practice), the volume brought to the 20 ml. mark and the whole again mixed.

A series of standards is simultaneously made up by taking 0·2, 0·4, 0·6 to 2 ml. of dilute standard sodium fluoride, giving a range of tubes from 2 μg. to 20 μg. fluorine at intervals of 2 μg. These are diluted with water, the same quantity of hydrochloric acid (2 ml.) and reagent (2 ml.) added and also made up to 20 ml. mark.

Standards and unknowns are allowed to stand, the rate of fading being noted by occasional inspection, and final colour matching completed after a few hours. It is best to take final readings the same day since slight colloidal precipitation of " lake " may occur on over-night standing. Rate of fading is proportional to fluorine and is very rapid with high quantities. If too rapid, lesser aliquots than 15 ml. of distillate are introduced. A preliminary test of the distillate, to obtain a rough sight estimate of the quantity of fluorine before putting up the standards, is advisable as a time-saving precaution.

The best reading points, with the quantities of reagent prescribed, lie between 8 and 16 μg. and for accurate work it is best to adjust the quantity of distillate to read between these standards.

Although standards are set up at intervals of 2 μg. reading between the tubes is easy and an accuracy of 0·5 μg. is readily obtainable. If necessary, a repetition, with standards varying by 1μg. around the 10 μg. region of best fading, ensures reading accuracy of 5 per cent.

Blank on Reagents.—Blanks on reagents must naturally be conducted at sufficient intervals to provide the requisite deduction in determination of unknowns, and check recoveries from known amounts of added fluorine should be frequently inserted to control uniformity of technique and the " personal equation." With good reagents the " total blank," including ashing mixture and acid, is small and should not exceed 2 μg. on 15 ml. of distillate. Even a blank of this magnitude, however, is a considerable fraction of a 10 μg. reading so that assessment of total " analytical error " should be made from trials with known quantities of fluorine. The " total error " can usually be kept within plus or minus 5 per cent., a degree of accuracy quite sufficient for all ordinary work involving very small real magnitudes of fluorine.

Limits of the Method.—Theoretically, the quantity of fluorine which is determinable can be made exceedingly low since the reading volume of 20 ml. is arbitrary and the whole scale of operations can be reduced by using narrower matching tubes and smaller quantities of reagents. Theoretically also, the distillate or the residue of it after an aliquot test, can be reduced to any desired point by evaporation in platinum basins, or in silica basins after *special preparations of surfaces* (preliminary stewing with acid and alkali).

In actual practice, however, the magnitude of the blank becomes a limiting factor. In concentrating the distillate the " deduction for blank " is, of course, also raised and results become inaccurate when the unavoidable blank begins to approach the total fluorine found.

Special Precautions.—The main precaution to be observed is avoidance of spraying over of traces of sulphuric acid during distillation. The presence of sulphate, even in very small quantities, markedly accentuates fading. Careful control during distillation is sufficient to guard against this, but if there is any doubt the distillate should be tested with barium chloride.

In this connection it may be mentioned that barium chloride does not interfere with the reaction so that in dealing with drinking waters high in fluorine but otherwise clean, it is often sufficient to make the determination direct, omitting distillation, by first removing sulphate with the minimum of barium chloride.

A second precaution which only comes into consideration in special cases, concerns the removal of free chlorine from the distillate in cases where peroxides are present in the ash after dry combustion. If the sample, for instance, contains appreciable quantities of manganese, the oxide and chloride in the ash may react with the sulphuric acid and traces of free chlorine may appear in the distillate. This can be obviated by adding 0·5 ml. of 10 per cent. sodium nitrite to the matching tubes (including the standards) and allowing to stand for a quarter of an hour before final addition of the zirconium alizarin reagent.

Speed of Procedure.—With one battery of four distillations, as shown in the photograph, a single operator can conveniently manage eight to twelve determinations a day, arranging for ashing samples between distilling operations, and preserving all eight distillates for one colour matching.

Later Modifications.—In the method as described above a magnesium ashing procedure was adopted because of the subsequent ease of dissolving the ash in sulphuric acid, but calcium ashing is also practicable and calcium oxide is more easily obtained free from fluorine when it is desired to reduce the blank and use the total 150 ml. distillate after concentration.

The variability in a blank of 2 μg. per 15 ml. distillate is about $\pm$ 0·5 μg. or 5 μg. on the whole distillate. On a 10 gramme sample of material this only introduces an error of $\pm$ 5 per cent. if the quantity of fluorine present is 10 p.p.m., but if it is only 1 p.p.m. the error is raised to $\pm$ 50 per cent. This presents no difficulty with samples of water, or with urines of which 100 ml. can be evaporated before ashing, but real difficulties arise where the size of sample is limited by other considerations. The only feasible procedure is then to reduce the blank, particularly by preparing a fluorine-free ashing mixture. For this, 200 grammes of reagent quality anhydrous calcium chloride, or its equivalent of the hydrated crystalline salt, are dissolved in 600 ml. of distilled water. 110 grammes of ammonium carbonate are dissolved separately, 55 ml. concentrated ammonia added, and the volume made up to 600 ml. To the boiling calcium chloride solution 20 ml. of the ammonium carbonate solution is added, the small precipitate filtered off and rejected. Most of the contaminant fluorine is co-precipitated and thus discarded. The process is repeated four times in all. To the final filtrate of residual calcium chloride, now freed from fluorine, is added the remainder of the ammonium carbonate (520 ml.) and the large precipitate thoroughly

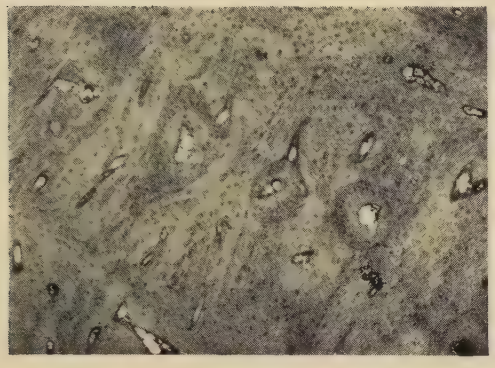

FIG. 5.

Cow 6.—Metatarsus, transverse section of compacta. × 50. Early case showing incipient porosis and uneven staining; irregular distribution of bone corpuscles. Some of the vascular canals show cartilaginous islets which appeared prominent in longitudinal sections.

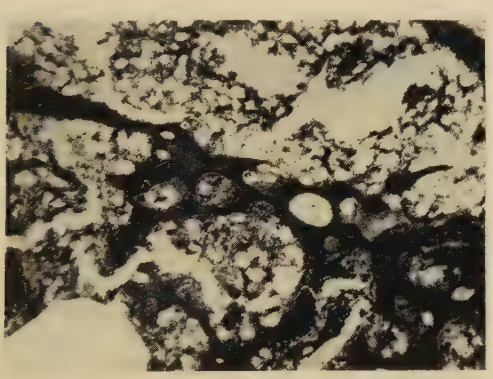

FIG. 6.

Cow 1.—Rib middle third, longitudinal section corticalis. × 25. Advanced case. Original compacta represented by slender porous trabeculae lined by osteoid tissue. Periosteum just visible at upper right hand corner. The spaces show remains of haemopoietic marrow.

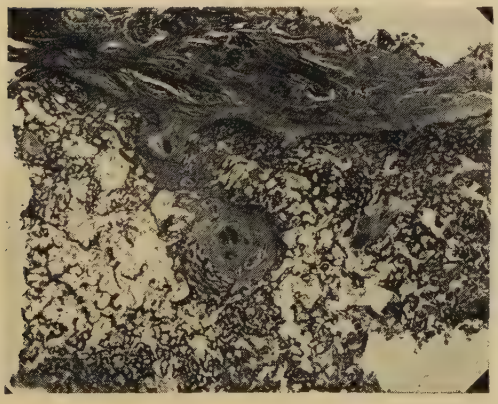

FIG. 7.

Cow 4.—Sternum. × 5. Advanced fluorosis showing marked resorption. The enlarged Haversian spaces resulting from thinning and disappearance of the trabeculae are occupied by loose fibrocellular or fibrillary material.

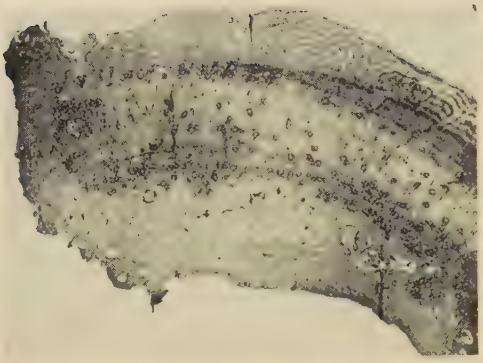

FIG. 8.

Cow 3.—Metatarsus, transverse section. × 5.
Various kinds of pores are seen throughout the compacta, the surface of which is covered by a diffuse periosteal deposit.

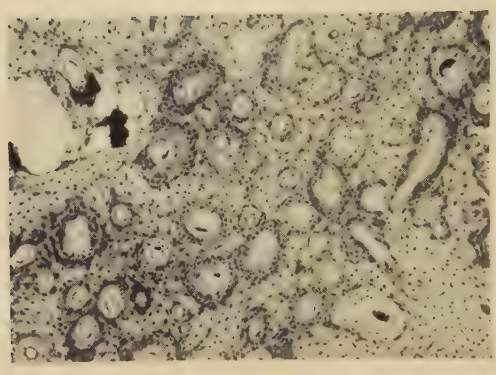

FIG. 9.

Higher magnification of preceding. × 50.
Showing distended Haversian canals, some of them lined by osteoid tissue. Uneven lamellation and marked irregularity in staining of the bony tissue.

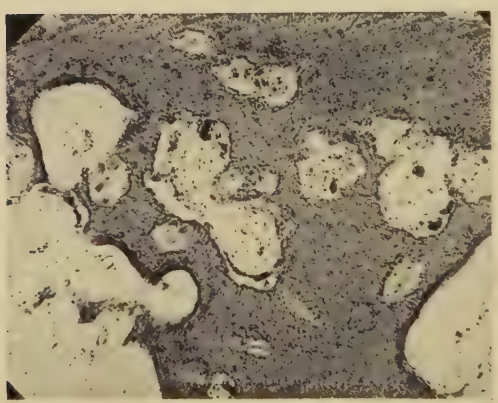

FIG. 10.

Cow 1.—Metacarpus transverse. × 60.
Absorption of bone by fibrocellular marrow; osteoclastic activity.

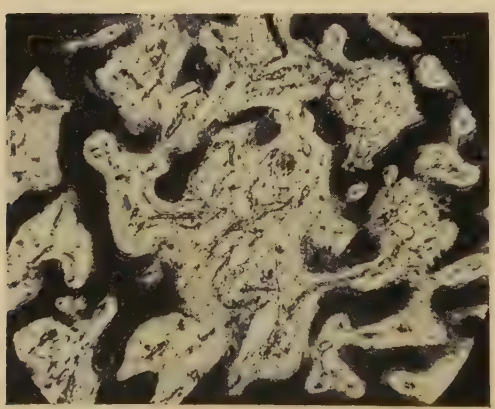

FIG. 11.

Cow 1.—Erostosis. × 30.
Showing typical resorption occurring in the deeper layers of newly formed periosteal bone.

washed, dried, ignited in an electric muffle, ground and stored as fluorine-free lime. Portions of this (1 gramme) can be used in ashing procedures, or a 10 per cent. suspension made up with water to which a little acetic acid is added to increase the amount of soluble calcium salt and so facilitate permeation of dry samples.

Using reagents purified as far as possible the blank can be reduced to 2 μg. in the whole 150 ml. distillate, which can then be evaporated to 15 ml. (or less), so increasing the sensitivity of the procedure ten-fold. Magnitudes of the order of 0·5 p.p.m., as in blood, can then be determined with an error of 0·05 p.p.m.

In recent years a refined thorium titration technique has been evolved, with Solochrome Brilliant Blue as internal indicator. As now used by Dr. Richardson (personal communication, 1947) of the M.R.C. Unit of Industrial Toxicology, it consists of (*a*) distillation of ashed sample with perchloric acid in presence of glass wool, in an " all glass " distillation unit, using silver perchlorate to precipitate chlorides and (*b*) titration of distillate with 0·004 N thorium nitrate in presence of monochloracetic buffer and solochrome brilliant blue, until the colour matches that of a control tube. Comparison of this method with our own earlier one gave no substantial difference in results, but it is superior in the sense that all titration methods are superior, and the end-point is sharper. Using a 2 ml. microburette the range 5 μg to 100 μg. is covered without adjustment of aliquots.

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THE PRODUCTION OF BOVINE TUBERCULOPROTEIN

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INTRODUCTION

In Great Britain, the tuberculin used for testing cattle is a purified protein derivative tuberculin, produced by the growth of three human strains (PN, DT and C) of *Mycobact. tuberculosis* on synthetic medium ; the technique of its production is described by Green (1946). While it is generally assumed that little difference exists between human and bovine tuberculin in their ability to detect bovine tuberculosis, it seems reasonable to suppose on general grounds that the homologous tuberculin would be preferable to the heterologous for use in the bovine, and this would certainly be the case if it can be shown that it is less likely to produce a reaction in avian sensitised animals, as suggested by the tests of McGirr, recorded by Green (1946).

The reason for the use of human strains in the production of P.P.D. is essentially a practical one, namely, that the yield of tuberculoprotein is usually high in glycerinated media compared with that from bovine strains. To remove this objection to the possible use of bovine P.P.D., work has been proceeding for some time at this laboratory to find if the yield from a bovine strain could be increased, or if a strain of bovine organisms, capable of producing large quantities of tuberculoprotein, could be obtained.

The latter possibility has been fulfilled in the study of a Weybridge strain of bovine tubercle bacillus, AN5, which is probably a eugonic variant of an originally dysgonic strain, and has been obtained on repeated subculture by a process of natural selection. AN5 grows well on the modified Sauton medium of Watson and Reid, but the yield of tuberculoprotein is small, resembling that of avian strains, and growth on this medium is therefore unsuitable for the production of P.P.D. tuberculin in large quantities. McTaggart, however, working in this laboratory, showed that AN5 would also grow in concentrations of glycerol higher than that of Reid's medium (6 per cent. glycerol) and could be grown on the medium introduced by the Bureau of Animal Industry (10 per cent. glycerol). AN5 in this latter medium produced tuberculoprotein in amounts comparable with that of the human strain DT on the same medium. McTaggart also pointed out that conditions conducive to development of an alkaline *p*H in the medium are also conducive to a high yield of P.P.D., and suggested that the customary steaming of cultures prior to filtration extracted protein from the human strain, while with the bovine strain the high yield of protein was present in the culture medium before steaming.

The present paper indicates the greater specificity of bovine tuberculin by means of tests on avian sensitised guinea-pigs, confirms on a larger scale some previous work for protein estimation, utilising a Kjeldahl technique instead of Hopkins' tubes, and indicates the reasons for the increased yields of bovine tuberculoprotein on BAI medium.

THE NON-SPECIFIC ACTIVITY OF BOVINE P.P.D. TUBERCULIN

Seibert and Dufour (1940) have pointed out certain anomalies in comparing results of tuberculin tests in guinea-pigs and in tuberculous human subjects, although both species are sensitive to concentrations of tuberculoprotein very low in comparison with that required to elicit a reaction in the tuberculous bovine subject. There is also some evidence to indicate that one animal species may reveal characteristics in a tuberculin different to those revealed by another species. Results on guinea-pigs may therefore be inapplicable to cattle, and it is realised that the practical value of the present observations remains to be tested on bovines under field conditions. Nevertheless, it seems probable that a tuberculin which has a low non-specific activity in guinea-pigs will also have a low non-specific activity in bovines if used in optimal dilutions in highly allergic individuals, and that inferior performance in the field is more likely to arise from limitations of field technique. Since it is not feasible to use a titration technique in routine tuberculin testing of cattle, it is necessary to use a standard test dose of high tuberculoprotein concentration, in order to ensure definite reactions in animals of low allergic status. When testing large numbers of individuals of unknown and widely varying allergic status, some of the properties of a more specific tuberculin may therefore be obscured or entirely lost. But if this is the case there would seem to be little hope of enhancing the specificity of field testing by improving the specificity of mammalian tuberculin and so obviating the need for " comparative tests " using more than one type of tuberculoprotein.

Methods

Biological.—In comparing the non-specific activity of human and bovine P.P.D. tuberculins, ten guinea-pigs were used in each batch tested. These were sensitised by intramuscular injection in the thigh of 0·5 ml. of a suspension of live avian organisms (14 day culture) of concentration 1 mg. organisms per ml. Three weeks after inoculation the guinea-pigs were depilated along each side of the back with clippers followed by barium sulphide paste and water. On the left side were injected intradermally 0·2 ml. of each of four serial dilutions of the standard tuberculin and on the right side the corresponding dilutions of the test tuberculin. The dilutions were arranged so that the highest just gave a slight area of inflammation and increasing strength increased the area of the inflammatory zone,

The diameters of these zones were measured in millimetres. The original strength of the test tuberculin was varied until the reactions of the test tuberculin matched the corresponding dilutions of the standard tuberculin.

Tuberculins.—These were prepared from P.P.D. powders, which were obtained by precipitating the tuberculoprotein from culture filtrate with trichloracetic acid, washing with dilute trichloracetic acid and drying with acetone and ether (Green). The powder was weighed on a microbalance, dissolved in M/15 Na_2HPO_4, the *p*H adjusted to 7·0 with M/15 KH_2PO_4 and an equal volume of a solution containing 20 per cent. glycerol (w/v) and 1 per cent. phenol (w/v) added. The final *p*H was checked and, if necessary, adjusted to *p*H 7·0. Dilutions of this solution were prepared with saline immediately before the test. The bovine P.P.D. was prepared from growth on B.A.I. medium, in distinction to the W.R. medium in the data reported by Green.

Results

The first test was carried out using human P.P.D. tuberculin as standard and bovine P.P.D. as test tuberculin. Preliminary trial showed that human P.P.D. at a strength 1·5 mgm. per ml. matched bovine P.P.D. at 4·5 mgm. per ml. The experiment was repeated to confirm this result, with the result shown below :—

TABLE I

AVIAN SENSITIZED GUINEA-PIGS

	Left side *Human P.P.D. 1·5 mgm. per ml.*				*Right side* *Bovine P.P.D. 4·5 mgm. per ml.*			
Dilution ...	1 : 25	1 : 50	1 : 100	1 : 200	1 : 25	1 : 50	1 : 100	1 : 200
Cage								
8	16	16	12	11	16	14	12	11
	16	16	10	7	16	13	11	11
9	14	12	6	6	13	13	10	8
	13	11	9	8	12	8	8	4
	15	8	6	6	11	11	8	6
12	16	16	12	8	17	16	14	10
	15	16	11	8	18	16	12	10
	12	12	8	4	12	10	8	4
15	16	13	16	12	16	14	12	10
	10	8	4	4	10	10	9	6
Sum (mm.)	143	128	94	74	141	127	104	80
$(Mm.)^2 \times 10^{-3}$	20·4	16·5	8·8	5·5	19·9	16·1	10·8	6·4

Comparing the sums of reaction diameters, or the areas indicated by their squares, in corresponding dilutions, it can be seen that the reactions to human P.P.D. are the same as those to bovine P.P.D. of three times the strength. This result means that in the dilutions tested, three parts by weight of bovine P.P.D. is required to produce the same reactions as one part by weight of human P.P.D. on avian sensitised guinea-pigs, or, conversely, that bovine P.P.D. has only one-third of the potency of human P.P.D. in non-specifically sensitised guinea-pigs. The areas of the skin reactions, as denoted by the squares of column totals, vary logarithmically with the protein concentrations.

A second experiment was carried out in the same manner on two batches of avian sensitised guinea-pigs to test the activity of bovine and human P.P.D. separately against avian P.P.D. as standard, instead of directly against one another.

Here the previous results are confirmed. Between two and three times the quantity of bovine P.P.D. as compared with human P.P.D. is required to match a standard quantity of avian P.P.D. tuberculin. Since bovine P.P.D. gives reactions as strong or stronger than human P.P.D. in guinea-pigs, sensitised to mammalian strains (Green, 1946), it would seem that bovine P.P.D. is more specific in the sense of disregarding avian infections.

TABLE II

AVIAN SENSITIZED GUINEA-PIGS

	Left side *Avian P.P.D. 0·5 mgm. per ml.*				*Right side* *Human P.P.D. 1·5 mgm. per ml.*			
Dilution ...	1 : 250	1 : 500	1 : 1,000	1 : 2,000	1 : 50	1 : 100	1 : 200	1 : 400
Cage								
5	16	12	12	8	12	10	6	6
	14	11	10	6	14	12	10	6
	16	12	10	6	14	11	10	4
11	16	14	12	10	18	16	15	13
	18	15	15	10	14	13	8	6
	18	14	13	10	16	14	12	10
13	17	14	13	11	13	12	9	8
	16	14	14	12	14	14	8	8
14	18	15	14	9	18	18	16	12
	18	15	12	10	15	12	11	9
	18	17	16	12	15	16	10	8
Sum (mm.)	185	153	141	104	163	148	115	80
$(Mm.)^2 \times 10^{-3}$	34·3	23·4	19·9	10·8	26·6	21·9	13·2	6·4

TABLE III
AVIAN SENSITIZED GUINEA-PIGS

	Left side *Avian P.P.D. 0·5 mgm. per ml.*				*Right side* *Bovine P.P.D. 4·5 mgm. per ml.*			
Dilution ...	1 : 250	1 : 500	1 : 1,000	1 : 2,000	1 : 50	1 : 100	1 : 200	1 : 400
Cage								
4	18	14	12	10	16	14	14	10
	17	16	14	12	17	17	16	12
6	16	13	11	9	14	12	10	8
	17	16	15	10	18	18	14	12
	14	11	11	8	18	15	14	12
7	11	8	4	4	11	10	8	6
	11	10	7	7	11	11	8	8
	13	12	6	6	16	11	10	8
10	15	14	10	10	14	13	12	10
	15	12	10	10	15	15	12	12
	17	16	11	11	17	17	16	15
Sum (mm.)	164	142	127	97	167	153	134	113
Mm.^{2} × 10^{-3}	26·9	20·3	16·2	9·4	27·9	23·4	18·0	12·8

THE PRODUCTION OF TUBERCULOPROTEIN BY AN5 ON BUREAU OF ANIMAL INDUSTRY (B.A.I.) AND ON WATSON-REID (W.R.) MEDIA

This experiment was carried out to compare the production of tuberculoprotein by AN5 on B.A.I. medium and W.R. medium and to gain some knowledge of the flask to flask variation. The work, as originally executed, utilised small volumes of medium (30 ml.), but on repetition this was found to be rather unsatisfactory as, after eight weeks' growth, losses by evaporation and adsorption left very little medium. The method of estimating the tuberculoprotein, also, by measuring the volume of centrifuged protein deposit after addition of trichloracetic acid, was found to give values varying between rather wide limits. Determination of nitrogen by micro-Kjeldahl on the precipitated protein was therefore adopted.

Material and Methods

The experimental media have the following compositions :

B.A.I. Medium

Asparagine, 14·0 g.
K_2HPO_4 (anhydrous), 1·4 g.
$MgSO_4 \cdot 7\ H_2O$, 1·5 g.
Sodium citrate, 0·9 g.
Ferric citrate, 0·3 g.
Glucose, 10·0 g.
Glycerol, 100·0 g.
Water to 1 litre
(No *p*H adjustment necessary)

W.R. Medium

Asparagine, 5·0 g.
KH_2PO_4, 2·0 g.
$MgSO_4 \cdot 7\ H_2O$, 1·0 g.
NH_4 citrate, 2·0 g.
NaCl, 2·0 g.
Ferric ammonium citrate, 0·075 g.
Glucose, 10·0 g.
Glycerol, 60·0 g.
Water to 1 litre (*p*H adjusted to 7·2 to 7·4 with NH_4OH).

100 ml. portions were charged into sterilised cotton-plugged 300 ml. Erlenmeyer flasks of resistant glass and autoclaved at 15 lb. for eight minutes.

Tuberculoprotein nitrogen or "P.P.D. nitrogen," was estimated by diluting 5 ml. culture filtrate with 4 ml. water in a Pyrex test tube, adding 1 ml. 40 per cent. trichloracetic acid, mixing well by inversion and standing in the cold store at 4° C. overnight. The flocculated protein was then spun down in the centrifuge at 2,000 r.p.m. for 15 minutes, and twice washed centrifugally with 1 per cent. trichloracetic acid to remove all non-protein nitrogen. A knife point of catalyst mixture and 1 ml. conc. H_2SO_4 was added and the whole combusted over a low flame for eight hours. The ammonia nitrogen in the digest was distilled with 50 per cent. NaOH in the usual way in the Markham distillation unit, the ammonia being collected in 2 per cent. boric acid and titrated directly with N/28 H_2SO_4 from a 5 ml. micro-burette using methyl-red brom-cresol-green indicator. It should be noted that the precipitated "protein derivative" contains small amounts of nucleic acid and polysaccharide and to express the product in terms of P.P.D., the factor 7 is used in place of the conventional 6·25 for pure protein; *p*H measurements were made with the glass electrode utilising a Marconi *p*H meter.

Results

Two series of 60 flasks each were set up, one series consisting of B.A.I., the other of W.R. medium. All flasks were sown with one flake from a 14-day old culture of AN5, also grown on B.A.I. medium and incubated at 37·5° C. for ten weeks. At weekly intervals six flasks were removed from each series and steamed for three hours in a steam steriliser and filtered. The volume, *p*H and protein nitrogen of the filtrate were then determined, the latter being done in duplicate for each flask. Protein or protein derivative is expressed as 100 ml. original medium, and also as the actual quantity in the volume of filtrate obtained.

The coefficient of variation (C.V.) of protein yields per 100 ml. medium per week is also given. (Table IV.)

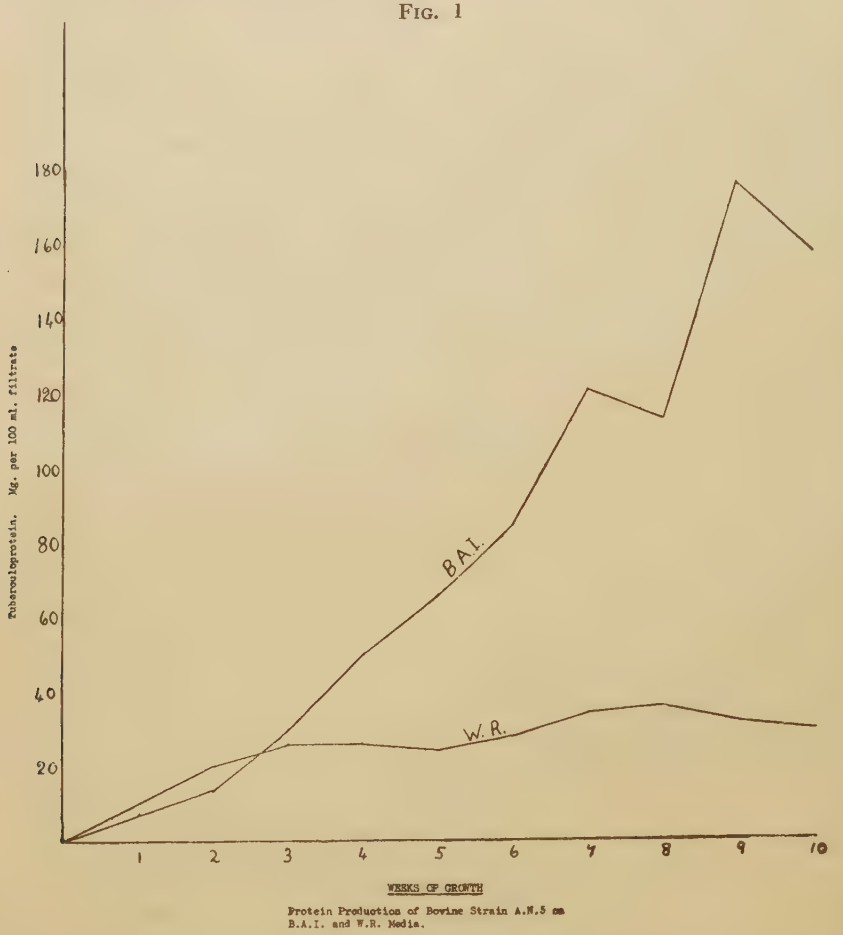
Table IV

Week	*Mgm. protein per 100 ml. Mean of 6 flasks*				*Filtrate volume. Mean of 6 flasks*		*Actual protein flask. Mean of 6 flasks*	
	B.A.I.		*W.R.*		*B.A.I.*	*W.R.*	*B.A.I.*	*W.R.*
	C.V.	*Protein*	*Protein*	*C.V.*	*Ml.*	*Ml.*	*Mgm.*	*Mgm.*
1	—	Nil	Nil	—	—	—	—	—
2	17·3	16	20	23·6	77	80	12	16
3	1·5	31	27	13·7	72	77	22	23
4	3·8	50	27	8·3	70	75	35	23
5	32·4	64	25	7·6	69	74	44	18
6–7	29·3	83	28	9·4	70	74	58	23
7	43·5	120	34	8·9	68	68	81	23
8	24·2	111	37	49·6	68	66	75	24
9	32·2	175	28	17·2	66	64	116	18
10	12·5	156	29	13·5	59	67	92	20

Fig. 1 clearly shows the greatly increased yields of tuberculoprotein obtained when AN5 is grown on B.A.I. medium.

After the third week of growth, AN5 on W.R. medium maintains an almost constant level of tuberculoprotein, while on B.A.I. medium a steady increase is obtained.

FIG. 1

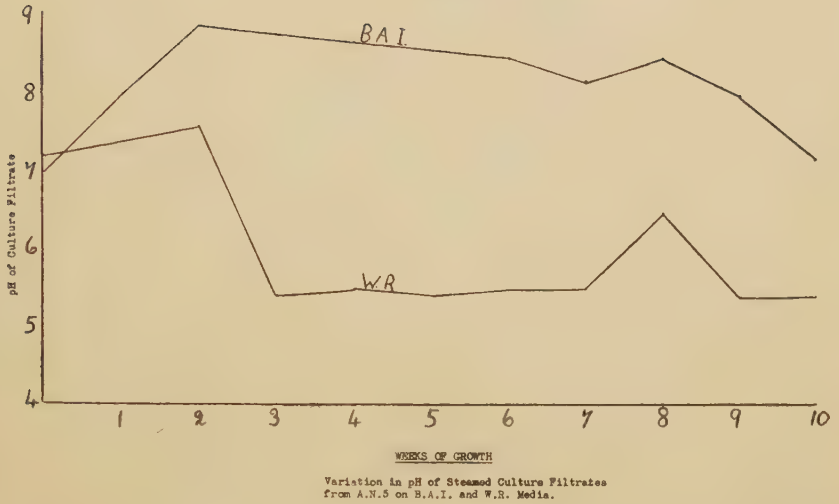


Protein Production of Bovine Strain A.N.5 on B.A.I. and W.R. Media.

Fig. 2 illustrates the variation during growth in *p*H values as observed after steaming. B.A.I. medium maintains a consistently alkaline *p*H while Reid's medium rapidly becomes acid in reaction. Again the flask to flask variation in *p*H was greater on B.A.I. medium than on W.R.

It is clear from the data presented that the bovine strain AN5 produces greater quantities of tuberculoprotein when grown on B.A.I. medium than when grown on W.R. medium, and that this increase is associated with an alkaline *p*H on the filtrates from steamed cultures.

FIG. 2



Variation in pH of Steamed Culture Filtrates from A.N.5 on B.A.I. and W.R. Media.

REASONS FOR INCREASED TUBERCULOPROTEIN PRODUCTION ON B.A.I. MEDIUM

During the cultivation of acid-fast organisms, the appearance in the medium of water-soluble proteins of low molecular weight, which are not coagulated by heat in the subsequent steaming process and constitute the active constituent of "tuberculins," has always been assumed (in this laboratory) to be due to autolysis of bacterial cells. Corper and Cohn (1943) have recently proved that this is the case. Working with washed bacterial cells from synthetic medium, they showed that tuberculoprotein production is the result of enzymic autolysis occurring best at *p*H 7·0 to 8·0. It therefore seems highly probable that the high yields on B.A.I. medium are produced because this medium maintains an optimum *p*H for autolysis. Several other possible factors, however, may also be predicted as responsible for the difference in yields on B.A.I. and W.R. media, as follows.

Growth on B.A.I. medium may produce a greater mass of organisms which give rise to a greater quantity of tuberculoprotein.

Steaming at alkaline *p*H may extract protein from the bacterial mass on B.A.I. medium.

Protein already formed on Reid's medium may be coagulated by steaming at an acid *p*H.

B.A.I. medium may maintain an optimum *p*H for action of autolytic enzymes.

Relationship Between P.P.D. Production and Bacterial Mass

Two series of flasks consisting respectively of B.A.I. and W.R. medium were sown with AN5, incubated at 37·5° C. and three flasks taken off at weekly intervals for eight weeks from each series.

After steaming, the organisms were filtered off on tared Whatman 50, 15 cm. filter papers, and the filtrate made up by washing the bacteria with cold water to 100 ml. volume. The bacteria were then thoroughly washed with hot water, drained, dried at 100° C. for 24 hours and weighed : *p*H and protein nitrogen were determined in the filtrate. Each week a single flask from each series was filtered raw through a Seitz E.K. filter, the bacteria being washed with about 20 ml. cold water and the filtrate finally made up to 100 ml. : *p*H and protein nitrogen were again determined in the filtrate.

Results.—These are shown in Table V. The mean values from three flasks each week were utilised in plotting Fig. 3, showing the correlation between P.P.D. production and weight of bacteria on the two media.

It is clear that a considerably greater mass of bacteria is formed on B.A.I. medium, especially after the sixth week, but that it is not the main factor responsible for the increased tuberculoprotein is shown by inspection of the protocols and by Fig. 3. The latter shows that equivalent masses of bacteria may produce very different quantities of tuberculoprotein, *e.g.*, at the eighth week 100 g. bacteria give 6 g. P.P.D. on B.A.I. medium and only 0·8 g. on W.R. medium. On B.A.I. medium alone, mean values show steadily increasing yields of P.P.D. from increasing weights of bacteria up to the seventh week, but individual values show considerable variation and no constant relationship between bacterial mass and P.P.D. prevailed.

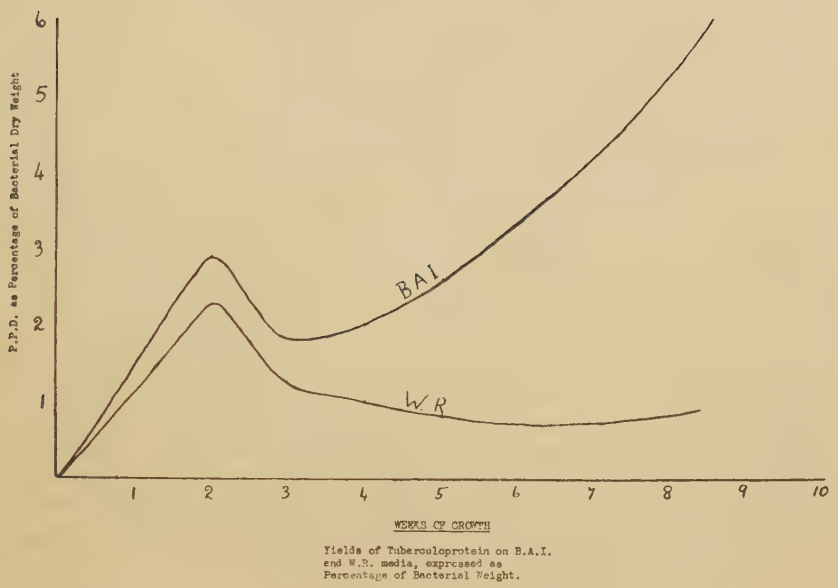
The most which can be said is that increased bacterial mass gives

TABLE V

	Week	*Steamed flasks (mean of 3)*					*Raw flasks (single)*	
		*p*H	*Protein (mgm. %)*	*C.V.*	*g. B.D.W.*	*C.V.*	*p*H	*Protein (mgm. %)*
B.A.I. Medium	1	6·8	—	—	0·04	50·0	6·9	—
	2	8·2	8	37·8	0·26	34·6	7·8	3·5
	3	—	15	8·9	0·84	13·1	—	1·8
	4	8·9	22	15·2	1·15	23·5	7·7	13·0
	5	8·9	36	24·6	1·35	50·4	8·2	19·6
	6	8·8	46	9·5	1·47	—	8·3	25·9
	7	9·0	70	26·1	1·73*	83·8	8·3	41·3
	8	9·1	91	34·3	1·53*	85·6	7·7	60·2
W.R. Medium	1	7·1	—	—	0·04	150	7·5	—
	2	8·8	11	3·8	0·44	682	7·8	Nil
	3	—	12	19·4	1·01	6·9	—	8·4
	4	5·1	11	11·3	1·17	2·6	6·0	11·6
	5	4·9	9	17·7	1·16	6·0	5·8	7·4
	6	4·9	6	12·2	1·15	3·5	6·3	4·9
	7	5·0	9	25·3	1·17*	6·0	5·0	4·9
	8	4·9	9	16·6	1·24*	3·2	4·9	7·0

* = Mean of 2 flasks. C.V. = Coefficient of variation.
B.D.W. = Bacterial dry weight.

FIG. 3



Yields of Tuberculoprotein on B.A.I. and W.R. media, expressed as Percentage of Bacterial Weight.

a greater quantity of P.P.D. if maintained under the correct conditions, probably those of *p*H. The character of the growth is a better indication of its P.P.D. production than the actual weight. On B.A.I. medium the bacterial mass tends to be expanded, thick, and fluffy while on W.R. it appears tight and granular.

Extraction of Protein by Steaming

Although tuberculoprotein remains in solution in slightly acid buffer solutions it is much more soluble in alkaline buffer solutions. It was thought that the steaming of cultures might be responsible for the higher yields obtained on B.A.I. medium, which terminates with an alkaline *p*H in contrast with Reid's medium, which maintains an acid *p*H throughout growth. Steaming at alkaline *p*H might extract protein from the cells and thus considerably increase the tuberculoprotein yield.

However, a number of facts militate against the significance of this factor. The yield of tuberculoprotein from raw cultures on B.A.I. medium is much higher than on W.R. medium (Table V) at all stages of growth, *i.e.*, a higher concentration already exists in the filtrate before steaming, and although steaming on B.A.I. medium may increase the yield of protein by 12 to 36 per cent., this protein can be shown to exist adsorbed, or very loosely combined on the surface of the bacteria and may be simply removed by washing the bacteria with distilled water. If a ten-weeks'-old culture of AN5 on 1 litre B.A.I. medium is separated from the culture fluid by

decantation and thoroughly drained, an equal volume of M/15 Na_2HPO_4 at *p*H 9 added and the whole steamed for three hours, the additional yield of protein thus obtained is considerable. One such flask yielded 570 mgm. protein in its raw filtrate and a further 430 mgm. protein in the phosphate extract. A similar flask, however, in which the bacteria were washed twice with two lots of 250 ml. distilled water yielded only 40 mgm. protein in the subsequent phosphate extraction. It seems evident, therefore, that the tuberculoprotein already exists as such in the bacillary mass and is not actively extracted from the bacteria by the customary steaming procedure at alkaline *p*H. The latter merely increases the filtrate protein by coagulation and contraction of the bacillary mass accompanied by dispersal of already soluble tuberculoprotein and very little conversion of insoluble bacterial protein to soluble form occurs.

Coagulation of Protein on Watson-Reid Medium.—Since the culture filtrate of W.R. medium lies on the acid side of 7·0 during the major period of growth (Table V), and is as low as *p*H 5·5 at the conclusion of steaming (Fig. 2), it seemed desirable to investigate the possibility of coagulation of protein being responsible for decreased yield. Aliquots of a raw filtrate of AN5 grown for ten weeks on B.A.I. medium were adjusted with 2N HCl to *p*H values ranging from the natural *p*H 8·3 of raw filtrate, to *p*H 4·0, at intervals of 1 *p*H unit, and all steamed for three hours in the steam steriliser. No coagulation of protein occurred at *p*H 8·3, 8·0, 7·0 or 6·0, but slight coagulation occurred at *p*H 5·0, the protein nitrogen of the filtrate being reduced from 0·88 mgm. per ml. to 0·50 mgm. per ml. At *p*H 4·0, 75 per cent. of the filtrate protein was coagulated, the drop in protein N being from 0·88 mgm. per ml. to 0·22 mgm. per ml.

The terminal *p*H of Reid's medium (Fig. 2), after steaming, lies at *p*H 5·5 approximately, *i.e.*, beyond the point where coagulation commences. The *p*H is generally nearer the neutral point before steaming and becomes more acid as steaming proceeds (Table V). Some coagulation may therefore occur on Reid's medium, but cannot be entirely responsible for the low yield, since, as shown in the previous paragraph, raw filtrates from B.A.I. medium contain significantly more protein than those from W.R. medium. Indeed, the small differences of protein per 100 ml. of steamed and of raw filtrate (Table V) suggest increased yields on steaming in Reid's medium, an effect probably due to dispersal of soluble protein from the bacterial mass.

B.A.I. Medium Maintains an Optimum pH for Autolytic Enzymes. Corper and Cohn have shown that tubercle bacilli grown on a protein-free synthetic medium for one month, washed, and autolysed in toluol at *p*H 7-8 at 37° C., produce a highly potent tuberculin after one week. The enzymes concerned are not inhibited by cyanide and hence do not contain a heavy metal. If their observations and

conclusions are correct, tuberculin of the culture filtrate is merely bacterial protein partly degraded by proteolytic enzymes of the bacterial cell. Many facts, of course, point indirectly to the autolytic production of tuberculin, notably its increase in the medium with increase of dead and dying cells in the bacterial mass, and decrease in weight of the latter at the time of maximum production of tuberculin.

The observed large increase in yield of tuberculoprotein by AN5 on B.A.I. medium can be, therefore, easily accounted for by the maintenance of an optimum *p*H for autolytic tuberculoprotein liberation and by no other fact. Other factors play only a minor role in the widely different yields obtained from B.A.I. and W.R. medium.

It is clear that any medium which is used for the production of tuberculin must be constructed so that a large bacterial mass is produced, and, equally important, an optimum *p*H simultaneously maintained for enzymic autolysis. A tuberculinogenic strain should, of course, also be employed, as differences exist between different strains of tubercle bacilli not only in their rate of growth but also in their ease of autolysis, presumably due to differences in the enzyme " battery " with which they are provided.

SUMMARY

Bovine P.P.D. from strain AN5 possesses only one-third the activity of human P.P.D. when tested on the avian sensitised guinea-pig. Since it is equally active on guinea-pigs sensitised with bovine strains, it should prove superior in detecting bovine infections, although this reasonable supposition remains to be tested under field conditions.

The glycerol-tolerant bovine strain AN5 consistently produces much greater yields of tuberculoprotein on B.A.I. medium than on W.R. medium.

The increased yields on B.A.I. medium result from the maintenance of an optimum *p*H for autolysis of bacterial cells, and are not merely the result of increased bacterial mass, or of greater extraction of organisms during the steaming process in the final stage of tuberculin production.

ACKNOWLEDGMENTS

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PROLIFERATIVE DERMATITIS OF THE LEGS ("STRAWBERRY FOOT ROT") IN SHEEP

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INTRODUCTION

This paper describes a form of dermatitis which affects the feet of sheep, and is known locally as "strawberry foot rot" (SFR). In many details, especially in clinical appearance, it closely resembles contagious pustular dermatitis (CPD) (Glover, 1928), but in others it presents important differences. In particular, SFR confers no immunity to CPD, and is unable to immunise even against itself.

The disease, which appears to be widespread in the North-East of Scotland, derives its name from the appearance of the lesions; if the covering scabs are removed a number of bleeding points present themselves on a fleshy mass and the result closely resembles a fresh strawberry. The condition is an ulcerative dermatitis affecting the skin of the legs from the coronet to above the knee or hock. If, as rarely, the horny structures of the hoof are affected, their involvement is the result of secondary bacterial invasion from the infected lesions. It is also rare to find the primary lesion in the interdigital cleft. SFR differs entirely from the foot rot of sheep, investigated and described by Beveridge (1935, 1938), which is a bacterial infection. Indeed, a reference has not been found in the literature which completely describes SFR. The important point is how far SFR can be distinguished from CPD, and this raises the question of how often the lesions of CPD appear on the feet and what form they assume in that situation. Among his five classes of "foot rot," Gregory (1939) noted a form of suppurative cellulitis affecting the area between the pastern and knee or hock, and a "podalic form" of contagious vesico-pustular dermatitis due to the same causal agent as the labial form. He found *Fusiformis necrophorus* in the lesions. Boughton and Hardy (1934), who published an account of CPD in sheep and goats involving some 6,700 cases, did not themselves observe lesions on the legs, although they recognised that such lesions could be part of the disease. Russell Greig (1943) stated that the legs were frequently affected in CPD and that the primary lesions were often situated in the hoof clefts, around the coronet and in the region of the fetlock. In severe cases, secondary bacterial invaders produced pus and caused ulcers and "raspberry-like masses" of tissue with ulcerated surfaces. Severe lameness might be caused by extension of suppurative processes under the horny tissue of the hoof. Glover (1928) found that it was only in severe outbreaks that the lesions of CPD spread from the lips to other parts of the body such as the coronet, where they rapidly increased in size to

form large fungoid masses which bled very rapidly. Newsom and Cross (1934), writing of CPD, stated, " . . . . leg ulceration, however, is a very rare lesion in our experience."

NATURAL DISEASE

The data in this paper are based on observations of the natural disease and of lesions produced by experimental inoculation of sheep. Many of the observations were made on a flock of 49 Blackface ewes bought in October, 1946, and still under observation at the time of writing (March, 1948). By day, this flock is grazed on common grazings on the rough hill pasture of Brimmond Hill, about one mile north of the Rowett Institute and about 800 feet above sea level. By night, the sheep are folded in a small holding paddock of some ten acres. When purchased in October, 1946, this flock was free from infection with SFR or CPD, and showed no old scars on the legs, such as would be expected in at least a proportion of the sheep if the flock had previously been infected with SFR. The sheep were placed on Brimmond Hill, which was known as infected ground, from 7.1.47 to 27.2.47. During this period, when there was severe, cold weather accompanied by heavy snow, and immediately afterwards, no cases were observed. The sheep were then transferred to clean ground on which they remained until lambing was completed.

On 10.4.47, after lambing, 42 of these ewes, together with 48 of their lambs, were returned to Brimmond Hill. Thereafter additions were made to the flock as follows : five ewes and five lambs on 22.4.47, one ewe and one lamb on 2.5.47, and one ewe and one lamb on 3.6.47. The exact date of the first case of SFR is not known, but at the weekly examination on 20.5.47, 38 of the 54 lambs, including the lamb introduced on 2.5.47, were found to be infected. The course of the infection in this group of lambs, as determined by weekly examinations, is shown in Fig. 1.

The incidence of the disease in these Blackface lambs increased sharply to reach a maximum seven to ten days after the first case was noted. The disease declined rapidly, thereafter, for about one month and reached a chronic stage which lasted several months and was marked by secondary invasion with pyogenic bacteria. Staphylococci and coliform organisms predominated among the bacteria isolated from such lesions. Throughout this period of nine weeks, as indicated in Fig. 1, the lambs were receiving treatment by external application of crude paraffin (kerosene oil). As it was thought possible that this might be unduly irritant and therefore responsible for maintaining the chronic stage of the disease, the treatment was stopped. Within two weeks the severity of the lesions had increased alarmingly and paraffin treatment was resumed ; in another two weeks the condition once more showed notable improvement. The sharp rise in incidence between weeks 11 to 12 (Fig. 1) coincided with the initial outbreak in the mothers of these lambs.

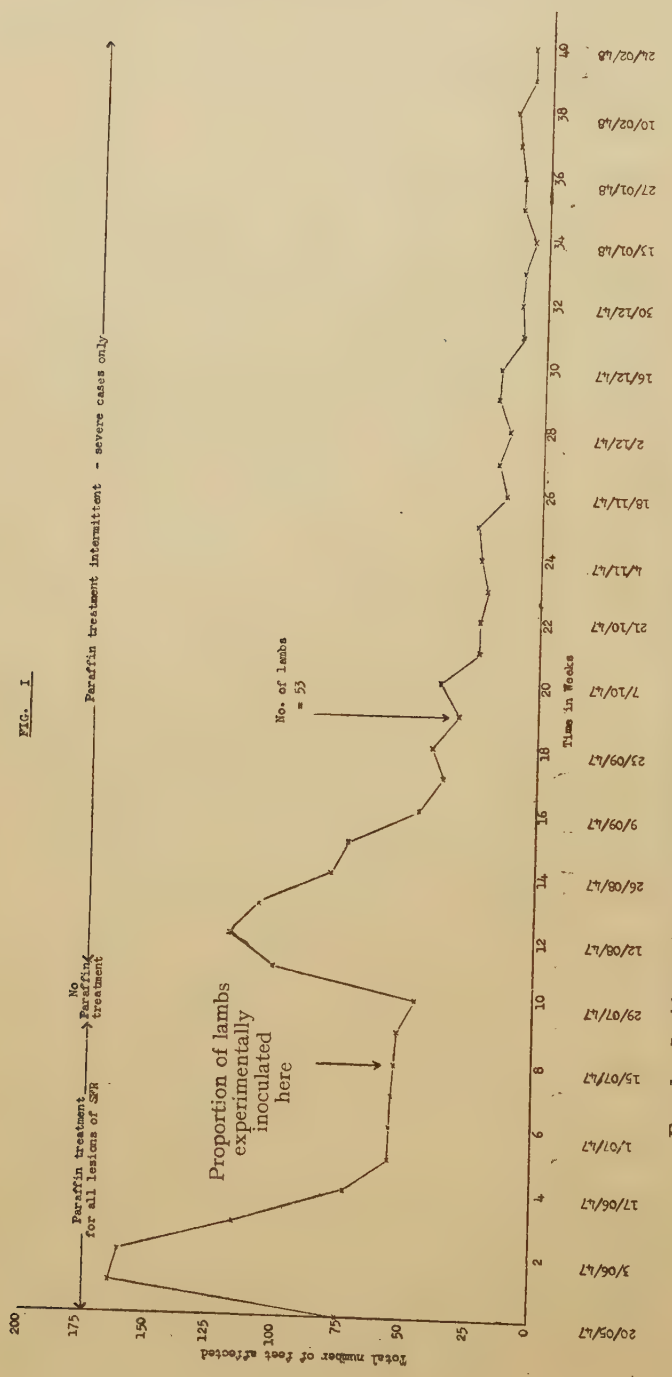

FIG. 1.—Incidence (No. of feet affected) of SFR in 55 Blackface lambs during 40 weeks.

Certain lambs, recovered from natural infection, had been experimentally re-inoculated two weeks before.

No case was observed in the 49 ewes until 5.8.47, when 28 of them were found to be affected. This was nearly four months from the time of their return to the infected pasture after lambing, eleven weeks after the initial outbreak in the lambs, and simultaneous with the second peak of incidence in the lambs (Fig. 1). In the ewes the disease rose sharply (Fig. 2), reaching a maximum at the end of a fortnight ; this was succeeded by a steep decline, almost as steep as the rise, and followed, as in the lambs, by a long period of chronic infection.

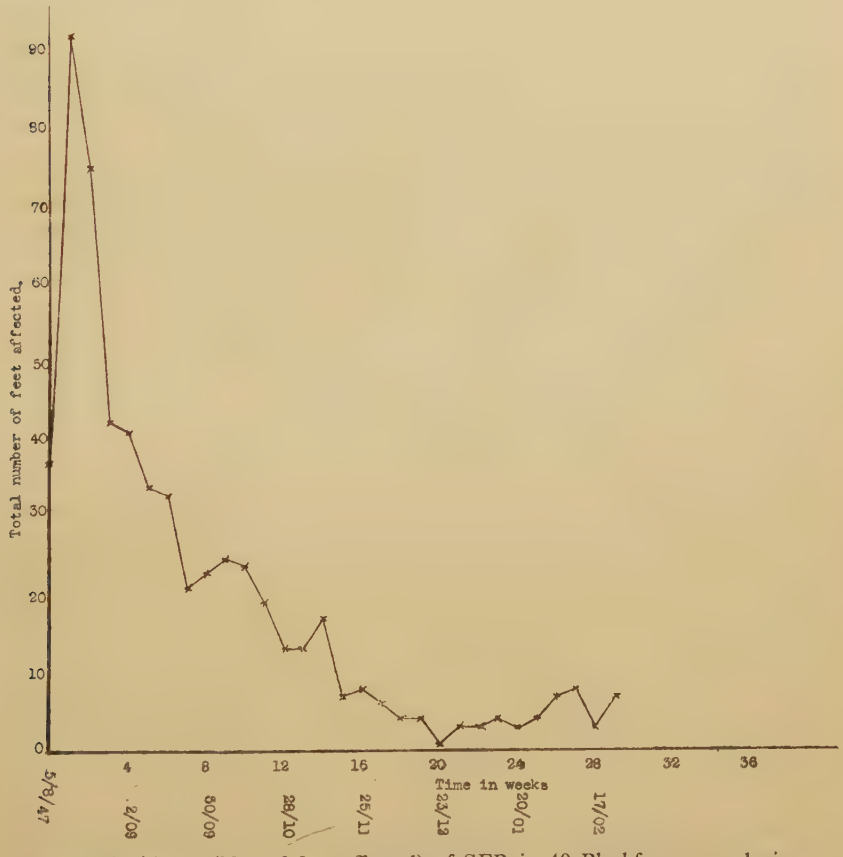

FIG. 2.—Incidence (No. of feet affected) of SFR in 49 Blackface ewes during 29 weeks.

Subsequently, at different dates, 37 Cheviot ewes, four ewes and four wethers of the Scottish Half-bred breed, 25 Cheviot lambs and two Half-bred X Suffolk lambs, were allowed to graze on Brimmond Hill. Observations on incidence, infectivity and course of infection among these groups of sheep are presented in Table I.

TABLE I

INCIDENCE AND COURSE OF SFR OUTBREAK

Group	Breed	Sex	Age	Animals infected			Feet infected		Date of first exposure to infection	Infection first noticed (days after exposure)	Total period of exposure (months)	Highest incidence (days after exposure)
				No. exposed	No. infected	Incidence (per cent.)	Total no.	Incidence (per cent.)				
I	Scottish Blackface	♂	Lambs	29	29	100	113	97	10.4.47	40	10	47
		♀	Lambs	26	26	100	102	98	10.4.47	40	10	47
II	Scottish Blackface	♀	2 years	49	49	100	142	72	10.4.47*	117	10	124
III	Cheviot	♂	Lambs	12	6	50	9	19	2.9.47	28	2	56
		♀	Lambs	13	13	100	43	83	2.9.47	28	5	100
IV	Cheviot†	♀	2 years	24	23	96	68	74	20.5.47	28	5½	28
				3	3	100	8	66	24.6.47	14	4	42
				10	8	80	15	38	5.8.47	14	2½–3	—
V	Cheviot	♂	2 years	1	1	100	1	25	Not known	Not known	Not known	—
VI	Half-bred (Cheviot × Border Leicester)	♀	2 years†	4	4	100	10	63	24.6.47	21	4	42
		wethers	2 years†	4	4	100	14	87	24.6.47	14	3¼	49
VII	Scottish Half-bred × Suffolk Cross, 1946	♂ & ♀	Lambs	20	13	65	18	22	6.9.46	21	⅔	Unknown (lambs sent for slaughter)
VIII	Scottish Half-bred × Suffolk Cross, 1947	♂	Lambs	2	2	100	7	87	5.8.47	98	3⅔	119

Also on infected pasture from 7.1.47 to 27.2.47 during heavy snow fall ; no case during this period. †These sheep were infected the previous year.

It will be noted that sheep grazed on Brimmond Hill are almost certain to become infected in one or more limbs. The numbers of the total limbs infected are : 97 per cent. in the lambs (214 out of 220), and 72 per cent. in the ewes (142 out of 196).

Since incidence alone might be an insufficient guide to the severity of the condition, a system of " points " was adopted giving each limb one, two or three points according to the severity of the lesions found upon it. For both ewes and lambs the results showed no appreciable difference from those for incidence alone and have therefore been omitted. Sheep of all ages are susceptible, though lambs appear to contract the disease more readily than older sheep. The disease appears to affect both sexes with equal severity. The four breeds of sheep exposed to infection—Scottish Blackface, Cheviot, Scottish Half-bred, and Half-bred X Suffolk Cross—all proved highly susceptible. The most severely affected animals—two in number—were of the Half-bred X Suffolk Cross breed.

Severe outbreaks have been observed from May to November depending on the date of initial exposure to infection. Frost or a general fall in temperature appears to exert a favourable influence in preventing infection and in terminating chronic infection.

The lesion

The natural disease begins with a small heaped-up scab situated anywhere on the leg from the coronet to the knee or hock. The most usual site is in front of the coronet or in the region of the pastern joint. No papular stage has been observed. These initial lesions are sometimes so small as to support the local idea that infection results from inoculation from a prick by a bract of whin. In this connection it was of interest to observe that sheep in an adjacent field, where no whins grew, did not develop SFR although separated at some places from infected animals by only a wire fence.

Soon the adjacent skin becomes scaly and develops rough wart-like scabs, which may spread over a considerable area till the whole limb up to 1 to 2 inches above the knee or hock is involved (see Fig. 3). More usually the lesions reach the size of 2 to 4 cm. in diameter before resolution takes place. The area involved rapidly becomes denuded of hair or wool. When large areas of the leg are affected the exudate which is formed mats the hair or wool and forms a firm, hard casing over the area. It is common to find that large areas of skin can easily be stripped by moderate traction on a few hairs or tufts of wool. It is rare for the disease to progress far into the woolled areas above the knee or hock. Natural infection on the face or the woolled parts of the body has not been observed.

Individual lesions may persist for long periods—up to 25 weeks in one case—but most heal completely in five to six weeks. Excessive pus formation is unusual but persistent cases commonly degenerate into lesions containing much greenish yellow pus, and some later show adherent papillomatous outgrowths. In CPD, Glover

(1928) described dense wart-like outgrowths with a superficial resemblance to a papilloma, which persisted for several weeks but were eventually resorbed.

Removal of the scabs before secondary infection is evident reveals a shallow ulcer circumscribing a raw surface with numerous bleeding points. Later, this ulcerated area becomes deeper, more pronounced and filled with pus. At this stage bleeding points are not seen.

FIG. 3.—Strawberry-like lesions on the feet of a lamb after removal of the scabs.

This secondary infection shows little tendency to invade the deeper structures of the leg. Lameness is rare and appears only if the interdigital space becomes infected.

Detachment of the scabs appears to cause considerable irritation but undisturbed lesions do not provoke either biting or scratching. The infection is unaccompanied by systemic disturbance, but severely infected sheep fail to make normal gains in weight. When the rough, wart-like scabs fall off, the underlying skin is scaly at first but hair and wool grow again. A scar is not formed unless secondary infection has supervened and pus formed.

Incubation period

Under natural conditions sheep and lambs may be found with the disease in about two to four weeks after being put on infected pastures (Table I). The longest intervals between exposure and appearance of the infection were 98 days for lambs and 117 days for adult sheep.

EXPERIMENTAL DISEASE

Transmission experiments

The infection was readily transmitted to sheep by scarifying the skin (see section on immunity). Attempts made to transmit the disease by rubbing infective material into the scarified skin of rats,

mice, guinea-pigs, and rabbits of Dutch or mixed breeds were unsuccessful. Selbie and Robinson (1947), however, report success with this procedure in rabbits. Man is susceptible, as was proved when inoculation of the hand resulted in a scab-like lesion at the site of infection which itched intensely from the fourth to ninth days and persisted for 30 days. A dried scab, taken when it was ready to fall off and inoculated by scarification into the skin of a sheep, reproduced typical lesions of SFR.

Titration of infective material

To prepare infective SFR material, scabs stored in glycerol-saline at 0° C. to 4° C. for from three days to 12 months were weighed, macerated in sand and suspended in normal saline to give suspensions (w/v) in a range of from 1 in 10 to 1 in 10^6 by intermediate steps that differed in different experiments but were usually tenfold dilutions or closer. These dilutions were inoculated by scarification into the cleaned skin of the inner side of both thighs. Four different experiments were made, each on from one to three sheep, eight animals being studied in all. All these sheep originally belonged to a flock in which SFR had been widespread the previous year and most showed traces of previous infection in the form of healed scars.

Recent and stored material did not differ greatly in infectivity and typical positive takes were produced by dilutions up to 1 in 500,000.

Mode of spread

The exact mode of spread has not been closely investigated. As with CPD, the dried crusts are highly infective for long periods and their presence on infective pasture is the probable source of fresh outbreaks, infection being introduced through skin abrasions and cuts. The frequency with which the coronet and knee are involved suggests that infection may be acquired in these sites while the sheep is resting. As shown in Table II, the hind legs are more often infected than the forelegs.

TABLE II
INCIDENCE OF SFR INFECTION FOR DIFFERENT LEGS

Breed	*No. of sheep*	*Legs infected*			
		Fore		*Hind*	
		Left	*Right*	*Left*	*Right*
Cheviot ...	45	24	23	35	30
Blackface ...	49	33	30	40	36
	94	57	53	75	66
Totals		110		141	

Hind legs *v.* fore legs $\chi^2 = 12{\cdot}2$ (significant)
Right legs *v.* left legs $\chi^2 = 2{\cdot}1$ (not significant)

Nature of infective agent

According to Blakemore* (1947), the causative agent will pass through 0·69μ gradocol membranes. It is difficult to prepare active filtrates through Seitz SB discs—an observation in line with that made for CPD virus by Glover (1928), Manley (1934), and Selbie (1945), all of whom found that CPD was largely withheld by bacterial filters. The agent of SFR remains viable in dried crusts kept for over one year at 0 to 4° C.

Histology

Material for histological examination was obtained from the region of the coronet either from recently slaughtered lambs or by excision from sheep under anaesthesia.

The essential change is a proliferation of the Malpighian layer of the epidermis, with degeneration of the superficial cells, accumulalation of exudate on the free surface, leucocytic infiltration of the dermis and epithelium and a general inflammatory reaction throughout the affected area. Mitoses are readily found in the proliferating epithelium, which may increase in thickness up to 2,500μ compared with 20μ to 60μ for normal skin adjacent to the lesion. The hair roots and deeper parts of the follicles are not involved. Vesicle formation has not been observed, but the surface layers of the epithelium may show ballooning, infiltration with leucocytes and separation of intercellular connections. In well advanced lesions the stratum granulosum loses its tendency to stain deeply with haematoxylin and eosin, and eleidin granules can no longer be demonstrated. Some of the cells of the rete Malpighii contain bright eosinophilic masses which are probably keratin. Inclusion bodies are not seen.

Open lesions show a variety of Gram-positive and Gram-negative organisms in the exudate, but these appear unrelated to the epithelial change except in the presence of general breakdown of the lesion with ulcer formation. The larger hyperplastic columns of epithelium when cut transversely show in some cases typical cell nests and resemble an epithelioma rather than a papilloma, although they are clearly not part of a malignant growth.

The structure of the lesions is identical with that described by Glover (1928) for CPD in the sheep. Involvement of hair sheaths, mentioned by Selbie (1946) in the rabbit, was not seen. During regeneration, the hyperplasia of the rete Malpighii diminishes and shows a tendency to return to normal, unless scar tissue is formed.

Immunity

Eight half-bred ewes, bedded indoors on fresh sawdust, were inoculated with infective material prepared by macerating dried scabs in normal saline, centrifuging and using the supernatant fluid

* Personal communication.

as inoculum. All these ewes, which were purchased in the open market in the previous year, bore on their legs numerous scars similar to those observed in animals that have recovered from SFR which has been followed by suppuration. The skin was shaved one to two days before inoculation, cleaned with ether and an area about 1·5 cm. in diameter was scarified with the point of a sterile needle. The inoculum was dropped on to the scarified area and rubbed over it with a sterile spatula. During the next three to four days the scarified area appeared to be healing, but on the fourth day wart-like growths appeared in the region. By the fifth day these grew together and increased to form an area about 3 cm. in diameter by the tenth day. These lesions, which appeared in all the eight animals inoculated, persisted with little change and without pus formation for from 31 to 62 days ; they were indistinguishable from those associated with the natural infection. This observation led to a test of the immunity conferred by an attack of SFR against subsequent infection. On 15.7.47, within two months of the initial (1947) outbreak in Blackface lambs (Group 1, Table I), ten males and nine females, which had been severely affected with SFR but had completely recovered one to three weeks previously, were inoculated with material from dried SFR scabs by scarification of the skin on the coronet of the left foreleg and the left commissure of the lips. Successful takes resulted in 18 of the 19 animals inoculated (Table III).

A second attempt at reinfection was made with 14 male and 15 female lambs, all of which had recovered either from a severe natural infection or from a severe reaction to experimental inoculation some four months previously. The test inoculum was macerated SFR scabs which had been passed through a Seitz SB disc and proved free of bacteria. Lesions resulted in 22 of the 29 animals, but in most lambs they were scanty and showed little tendency to spread from the scarified area.

Cross-immunity Tests.—The close resemblance between the lesions of SFR and CPD indicated the need to compare the immunological properties of the infecting agent from the two types of lesion. Accordingly, SFR material from the Blackface flock was compared with virus obtained from typical CPD of the lips in a Cheviot flock which was housed indoors at the Rowett Institute and had no contact with the SFR-infected Blackface flock. Unfiltered material from the CPD lesions was inoculated by scarification into the right foreleg and right commissure of the lips of 12 male and eleven female lambs, 19 of which were inoculated simultaneously with SFR. The results (Table III ; 15.7.47) showed that :—

1. Lambs only recently recovered from a severe infection with SFR could readily be reinfected about the legs ; the resulting lesions were usually as severe as those of the naturally acquired disease and persisted for more than six weeks.

TABLE III

EXPERIMENTAL INOCULATION OF BLACKFACE LAMBS RECENTLY RECOVERED FROM SFR

Date	15.7.47				5.8.47		29.10.47			
Inoculum	*SFR (unfiltered)*		*CPD (Rowett, low virulence; unfiltered)*		*CPD (Rowett, low virulence; unfiltered)*		*SFR (Seitz-filtered)*		*CPD Cambridge, high virulence; unfiltered)*	
Site	*Left coronet*	*Left lip*	*Right coronet*	*Right lip*	*Right coronet*	*Right lip*	*Left coronet*	*Left lip*	*Right coronet*	*Right lip*
Lamb No.										
402	+++	+	++	—			—	—	++	+
403	+++	—	—	—			+	—	+++	+
405	...	...	—	—						
406	+++	+++	+	—						
407					+++	+				
410					++	+				
411					+	—	—	+	+	+
412					++	—				
413	...	...	+	—						
414	+++	+++	+	+						
416	+++	+++	—	—			—	—	++	++
417							—	+	+++	+
418	+++	+++	—	—						
419	+++	+++	+	—						
420	—	—	—	—			+	+	+++	+++
421							—	—	++	+
422	+++	+	+	—						
423							+	+	+	+
424							+++	+	—	+
425							—	+	+++	++
426	...	...	+++	—						
427							++	+	++	+
429	+++	+	—	—						
430					+	+				
432							+	+	+++	+
433							+	+	+++	++
434					+	+				
435	...	...	+++	—			+	+	+	+
436					+	+	—	+	++	++
437					+	+				
439							+	—	+++	+++
441							+++	—	+	+
442	++	+++	—	—						
444	+++	—	+++	—			+	—	++	++
445	+++	+++	+	+						
446							—	—	+	+
447							—	+	+	+
448					+	—				
450							—	—	+++	+
451	+++	+++	+++	—						
452					+	+				
453	+++	+++	—	—			—	—	++	+
454					++	—				
456					—	—				
457	+++	+++	+	++						
459							+	+	+	+
460							+	+	++	+
461							+	+	+++	+
464							+	—	++	+

TABLE III—*continued*

Date	15.7.47				5.8.47		29.10.47			
Inoculum	*SFR (unfiltered)*		*CPD (Rowett, low virulence; unfiltered)*		*CPD (Rowett, low virulence; unfiltered)*		*SFR (Seitz-filtered)*		*CPD Cambridge, high virulence; unfiltered)*	
Site	*Left coronet*	*Left lip*	*Right coronet*	*Right lip*	*Right coronet*	*Right lip*	*Left coronet*	*Left lip*	*Right coronet*	*Right lip*
Lamb No.										
465	+++	+++	—	—			—	—	+	+
469	+++	—	—	—						
470							—	+	+++	+
471	+++	+++	—	—			+	++	+++	+
Totals: Takes / Inoculated	18/19	15/19	12/23	3/23	11/12	7/12	16/29	17/29	28/29	29/29
Analysis of takes										
+++	17	12	4	0	1	0	2	0	11	2
++	1	0	1	1	3	0	1	1	9	6
+	0	3	7	2	7	7	13	16	8	22
—	1	4	11	20	1	5	13	12	1	0

+++, ++, + = degrees of extent and severity of lesions.
— = no lesion after inoculation.
... = no inoculation into this site.

* On 15.7.47 and 5.8.47, the lambs were returned to infected pasture after inoculation and the sites of inoculation were not protected; it is thus uncertain if the coronet lesions were CPD or SFR. On 29.10.47 the animals were not returned to infected pasture after inoculation and the sites of inoculation were covered with gauze.

2. Inoculation of SFR into the lip might be successful, but lip lesions were always much smaller than those on the coronet, were rapidly regressive, showed no tendency to suppuration, and healed within two or three weeks.

3. A Rowett Institute strain of CPD inoculated on to the right coronet and right lip failed to produce lesions of typical extent or severity (Table III; 15.7.47)—a failure which was considered as explained by low virulence of the strain, CPD. In this experiment the nature of the lesions at the site of CPD inoculation in the coronet region is uncertain because the inoculated lambs continued to graze on SFR-infected pasture and may have acquired this infection through the inoculation site.

A second inoculation of the Rowett Institute strain of CPD was made three weeks later. Again lambs recovered from SFR were

used but no simultaneous inoculations with the latter were made. The results (Table III ; 5.8.47) produced more numerous takes on the lip than in the previous experiment ; the results of leg inoculation were again of uncertain significance because the animals were returned to SFR-infected pasture after inoculation.

Two and a half months later a further attempt was made to compare the infecting agents of SFR and CPD. For this experiment the SFR material was reduced in potency by filtration through a bacterial filter. A known virulent strain of CPD virus, obtained through the courtesy of Mr. F. Blakemore, Institute of Animal Pathology, Cambridge, was used without filtration. In order to prevent SFR infection being introduced naturally through the scarified areas after the lambs had returned to their infected grazing, both fore coronets were covered with sterile gauze and tightly bound with adhesive tape. Further, the inoculated sheep were not returned to their infected grazings for seven days. Although the adhesive tape did not remain in place for longer than three to four days, this period was sufficient since " takes " due to inoculation could be recognised at this time. The sites chosen were shaved five days before inoculation and only those were used in the experiment which remained clean and free from bacterial infection. Immediately before scarification the skin was cleaned with ether and allowed to dry. The results (Table III ; 29.10.47) indicate that lambs recovered from SFR are not immune to reinoculation with SFR or to simultaneous inoculation with virulent CPD.

In this experiment inoculation of the coronet with CPD virus resulted in lesions, but in this site they regressed more rapidly than those of SFR and were completely healed in three to four weeks. Lip lesions produced by the virulent Cambridge strain of CPD remained localised and healed completely in from three to four weeks. CPD was not transmitted from the inoculated to the uninoculated sheep, as might have been expected. These experiments on an SFR-infected flock on infected pasture did not permit the uncomplicated type of observation possible in laboratory animals or in individual sheep housed in uninfected quarters. They supported the observation that under natural conditions sheep can have more than one attack of SFR, which does not confer absolute protection against inoculation with virulent CPD.

DISCUSSION

The clinical and histological similarity of the lesions of SFR to those of CPD must tend to conceal the existence of SFR as a separate disease entity. It is important, therefore, to emphasise the differences which separate the two conditions. These are summarised in Table IV.

TABLE IV
DIFFERENCES BETWEEN SFR AND CPD

	SFR	*CPD*
Usual site	Legs	Lips
Usual age of infected animal ...	Any age	Lamb
Immunity to second attack ...	None observed	High
Cross-immunity tests	No protection against CPD	Protection against SFR not tested
Duration of uncomplicated lesion	30–35 days	14 days

Acceptance of SFR either as a separate disease or as an immunological variant of CPD might account for some anomalies in the results of preventive vaccination against CPD, about which Lyle Stewart (1948) states : " On eight farms the results were less satisfactory. These farmers reported no benefit from vaccination, the reported incidence being over 20 per cent., with a proportion of severe heel cases." The possibility that SFR was prevalent on these eight farms is consistent both with the poor results of vaccination with a CPD vaccine and the proportion of severe heel cases.

The observations recorded here show that SFR fails to immunise against itself and that recovery from an attack of SFR does not confer immunity against CPD. It remains to be tested whether immunisation with CPD confers immunity against SFR.

SUMMARY

A description is given of a form of dermatitis of the legs of sheep and lambs known in North-East Scotland as " strawberry foot rot " (SFR). The disease is characterised by haemorrhagic wart-like outgrowths on the legs. The morbidity rate on infected pasture is high, often 100 per cent. ; the clinical picture and histology of the lesions closely resembles that of contagious pustular dermatitis (CPD).

The disease was studied clinically and experimentally in 90 ewes, four wethers, and 82 lambs of several breeds over a period of 44 weeks. During this time there were two short periods of widespread acute infection ; otherwise the disease remained chronic with sporadic acute cases.

In the observations which form the subject of this paper, SFR conferred no immunity to CPD and one attack did not prevent a second.

Acceptance of SFR as a separate disease or an immunological variety of CPD might explain differences in the reported efficacy of CPD vaccine.

ACKNOWLEDGMENTS

I have pleasure in thanking Dr. F. R. Selbie, Bland-Sutton Institute of Pathology, Middlesex Hospital, and Mr. F. Blakemore, Institute of Animal Pathology, Cambridge, for infective material and information about their unpublished experiments; and Mr. M. H. Quenouille, Statistics Department, Aberdeen University, for analysis of the figures in Table II.

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THE IMMUNOLOGICAL RELATIONSHIP BETWEEN A NEW VIRUS, PROLIFERATIVE DERMATITIS OF THE LEGS ("STRAWBERRY FOOT ROT") OF SHEEP, AND THE VIRUSES OF VACCINIA AND CONTAGIOUS PUSTULAR DERMATITIS

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INTRODUCTION

In September, 1947, one of the authors (E.S.H.), when visiting Dr. Selbie, Bland Sutton Institute of Pathology, Middlesex Hospital, London, was informed that a new virus of the animal pox group, provisionally termed proliferative dermatitis of the legs, or "strawberry foot rot," from the appearance of the feet lesions, had been isolated from sheep in Scotland. Dr. Selbie kindly supplied some infective material consisting of dried skin scrapings from a rabbit inoculated four days previously.

On the author's return to Khartoum a series of tests were instituted. As the Stack Medical Research Laboratories are also a vaccine lymph institute, all the work was carried out in the Veterinary Research Laboratories a mile distant.

METHODS

Technique

Inoculations were made in rabbits, goats and sheep. The method of preparing the virus suspensions, etherisation and inoculation by scarification on to the shaved flanks or bellies was the standard technique used in these laboratories by Bennett *et al.* (1944). For storage purposes small portions of the skin pulp were kept in the freezing chamber of a refrigerator.

Viruses Used

Strawberry Foot Rot (hereafter referred to as SFR).—After scarification of the skin of the rabbit there was confluent redness and swelling on the second day. By the fourth day the lesions had developed to a honey-coloured, fibrinous sheet or confluent crust with numerous cracks which showed raw, bleeding areas underneath. On the edges of the area there were discrete, yellowish pustules which very rapidly dried, forming yellow crust not unlike human impetigo. The lesions commenced to dry up about the fourth day and resolution was rapid, the crusts falling off between the tenth and twelfth days. The appearances of the lesions on goats and sheep were similar. In addition to their more rapid evolution,

the lesions were readily distinguishable on all animals from those of vaccinia and contagious pustular dermatitis (CPD).

The dried virus received from Dr. Selbie was inoculated on to both flanks of a rabbit which was killed on the fourth day. The crusts were ground up in distilled water to make a thick cream, and the suspension was used for all the earlier immunising tests. Before carrying out cross immunity tests, fresh material was prepared from another rabbit. For the later sheep (7 to 15) the second rabbit passage virus was inoculated on to a third rabbit and the subsequent material used for both immunising and cross-immunity tests.

Contagious Pustular Dermatitis (CPD).—With one exception (sheep 11 which was immunised with a Cyprus strain No. 1), the standard English strain (UF) was used for all experiments. The appearances and evolution of the lesions on rabbits, sheep and goats were identical with those described in a previous paper (Horgan and Haseeb, 1947).

Vaccinia.—The standard strain used in these laboratories for preparation of vaccine lymph was used in all tests. In the case of both CPD and vaccinia, the infective material was derived from sheep.

RESULTS

Effect of Ether on the SFR Virus

In the earlier tests etherisation was carried out on the SFR material. It was noted, however, that there appeared to be a viricidal action of the ether which is demonstrated by comparing the reactions in rabbits 7 and 8 respectively (Table I). The alternative explanation of variation of individual susceptibility was also borne in mind and to decide the matter two rabbits, which are not shown in the table, were heavily inoculated on one side with non-etherised SFR material and on the other with some of the same suspension etherised for 12 hours. The results were conclusive ; both rabbits failed to show any reaction with the etherised material while the takes with the non-etherised were confluent.

Unfortunately, several cross immunity tests had been carried out before this viricidal action of ether was discovered, and hence the absence of takes with SFR virus in the cross immunity tests on rabbits 1 and 2, goats 1 and 2, and sheep 1, cannot be regarded as significant. In all the other tests, non-etherised suspensions of SFR virus were used.

Spreading of Lesions

In Table I a query has been placed after the readings of the CPD tests in sheep 3 and 4. The area inoculated with the test virus was adjacent to that inoculated with the SFR virus, and after the third day the latter had spread over most of the area of the former thus rendering later readings impossible. This curious tendency of

TABLE I

CROSS IMMUNITY TESTS BETWEEN VACCINIA, CONTAGIOUS PUSTULAR DERMATITIS AND STRAWBERRY FOOT ROT VIRUSES

	Immunising Strains			*Test Strains*			
Animal No.	*Immunising virus*	*Date immunised*	*Result of immunisation*	*Date tested*	*Vaccinia*	*CPD*	*SFR*
Rabbits							
1	SFR	29.9.47	++++	9.11.47*	++++	++++	O
2	„	7.10.47	++++	9.11.47*	++++	++	O
3	„	17.11.47	++++	2.12.47	++++	++	++
4	„	17.11.47	++++	2.12.47	++++	++	++
5	CPD	3.11.47	+++	2.12.47	++++	O	+++
6	„	3.11.47	+++	2.12.47	++++	O	++
7	Control			9.11.47*	++++	+++	O
8	„			2.12.47	++++	++++	+++
Goats							
1	SFR	7.10.47	++++	9.11.47*	++++	+++	O
2	„	7.10.47	++++	9.11.47*	++++	+++	O
Sheep							
1	SFR	7.10.47	++++	9.11.47*	++++	+++	O
2	„	17.11.47	++++	3.12.47	++++	++++	O
3	CPD	3.11.47	++++	3.12.47	++++	+ ?	++++
4	„	3.11.47	++	3.12.47	++++	+ ?	++++
5	Vaccinia	14.11.47	++++	14.12.47	O	++	++++
6	Control			3.12.47	++++	+++	++++
7	SFR	14.1.48	+++	7.2.48	++++	+++	O
8	„	14.1.48	+++	7.2.48	++++	+++	O
9	CPD (Glover)	14.1.48	+++	7.2.48	++++	+	++
10	„ „	14.1.48	+++	7.2.48	++++	+	++
11	„ (Roe)	19.1.48	+++	7.2.48	++++	+	++++
12	Vaccinia	18.1.48	+++	8.2.48	O	++++	++++
13	„	15.12.47	+++	8.2.48	+	++++	++++
14	Control			15.2.48	Not done	++++	++++
15	„			15.2.48	„	++++	++++

++++ = Confluent eruption.
+++ = Semi-confluent eruption.
++ = Scattered pustules.
\+ = Few abortive pustules drying to scabs by seventh day (" vaccinoid reaction ").
O = Absence of reaction.

* = Vaccinia, CPD and SFR test materials etherised.

In all other tests, non-etherised materials were used.

the SFR virus to spread was also noticed in the case of several other rabbits and sheep but as the areas were further apart the readings were not obscured.

Cross Immunity Tests

SFR Against Itself.—Excluding as doubtful the results of rabbits 1, 2 and 7, goats 1 and 2 and sheep 1, there remain rabbits 3 and 4 with control 8 and sheep 2, 7 and 8 with controls 6, 14 and 15. In all cases there was a good semi-confluent or confluent take of the immunising virus, and the absence of a reaction in the sheep tests indicated that complete immunity had been produced. On the

other hand, the results of the rabbit tests came as a considerable surprise as in the immunised animals the reactions were almost as strong as in the controls. The authors are unable to advance an explanation of what appears to be the inability of the virus to protect against itself. It seems scarcely credible that if any considerable degree of immunity had been produced by the immunising dose, it would have been completely lost in 15 days.

SFR and Vaccinia.—All the results are equally clear cut and consistent ; there was a complete absence of cross protection between the viruses indicating the lack of any immunological relationship.

SFR and CPD.—Allowing for slight variations in individual animals, the results showed no evidence of cross protection in either rabbits, goats or sheep.

SUMMARY

With the exception of the two rabbit tests, which at the moment are inexplicable, exposure to the virus of proliferative dermatitis of the legs in sheep (strawberry foot rot) induces an immunity against homologous infection. There is no evidence of any immunological relationship between the viruses of vaccinia, contagious pustular dermatitis, and SFR. The latter appears to be a new virus.

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SOME OBSERVATIONS ON PROLIFERATIVE DERMATITIS OF THE LEGS "STRAWBERRY FOOT ROT" OF SHEEP

By

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INTRODUCTION

In November, 1946, Mr. S. T. Harriss, of the Rowett Institute, Aberdeen, drew attention to an eruptive skin disease of sheep in Scotland, and kindly supplied dried scabs from natural and experimental cases. Clinically, the eruptions were described as similar to those of contagious pustular dermatitis (CPD) except that they were mainly restricted to the feet and legs and only rarely affected the nose and lips.

At this Institute, the disease was reproduced in sheep and goats as well as in rabbits and guinea-pigs. The infective agent was shown to pass through coarse gradocol membranes but was not visible in electron micrographs of infective material. In a limited number of cross-immunity tests, sheep recently recovered from CPD showed some resistance to strawberry foot rot (SFR), but the immunity did not persist for as long as it did against CPD itself. In rabbits it was not possible to show any cross-immunity between these two conditions.

TRANSMISSION EXPERIMENTS

Transmission to the Rabbit

The original material was suspended in McIlvaine's buffer solution (*p*H 7·0) and inoculated by scarification on the plucked skin of a rabbit. Five days afterwards, the skin became congested and showed numerous papules which by the following day were covered with thick yellowish white scabs. A week after the first appearance of the lesion, the scabs dried and fell off in the form of thick brittle pieces, leaving a thickened bare area. The lesion was appreciably more severe than is the case in CPD in the rabbit.

When serially transmitted in four rabbits the incubation period dropped from five to two days in the second passage and remained constant thereafter. Passage also increased the severity, but did not alter the character, of the lesions and healing generally took place in two weeks from the time of infection. Material from the fourth rabbit passage produced the disease in sheep and goats.

Histology of the Rabbit Lesion.—Pieces of infected skin were excised under anaesthesia or removed from freshly killed rabbits on the second to the eighth day after infection and fixed in Heidenhain's "Susa" mixture. Delafield's haematoxylin and eosin were chiefly used for staining.

Two-day lesions showed a slight proliferation of the epidermis, individual cells of the Malpighian layer were enlarged and in the basal layer some cells showed vacuolation around the nucleus. Reticular degeneration of the more superficial cells, which is a feature of CPD, was not seen. The dermis was markedly oedematous, congested and infiltrated with polymorphonuclear and mononuclear inflammatory cells. In two- or three-day-old lesions the dermal oedema had subsided but the epidermal proliferation was more marked. There was also thickening of the Malpighian layer, both under the skin surface and around the hair follicles, following an increase in the number of cell layers and swelling of individual cells. Mitotic figures were frequently seen in the nuclei of the deeper cells, but there was no reticular or balloon degeneration and inclusion bodies were not seen (Fig. 1). Necrosis of the superficial cells of the proliferated epidermis began about the fifth day proceeding from the superficial layer inwards and stopping at about the middle. In the next two days the necrosed layer (scab) became thickened with exudate and infiltrated with migratory cells (Fig. 2). By the eighth day, healing by regeneration of the epidermis was already in evidence. A noticeable feature of the onset of necrosis was the appearance in the dermis of a large number of eosinophil leucocytes which persisted in subsequent stages of the disease.

Transmission to the Goat

Two adult goats were infected with material from the rabbit and the lesions were more severe than those observed in the experimental disease of sheep. Both animals developed diffuse erythema and papulation of the skin in two days and the lesions became covered with a yellowish white scab which cracked, exposing the raw skin underneath. Removal of the scab from a part of the eight-day-old lesion revealed an area studded with discrete nodules. Crusts from one of the goats set up the typical disease in rabbits and sheep. A recovered goat was reinfected 35 days after the first infection. It developed a mild "accelerated" reaction lesion which healed in ten days.

Transmission to the Guinea-pig

Two guinea-pigs, inoculated by scarification with infective material derived from the rabbit, developed lesions resembling those of the rabbit after an incubation period of two days. The lesions were, however, discrete and healed in ten days in one case and 13 days in the other.

Experimental SFR in Sheep

Sheep inoculated by scarification on the plucked skin of the flank showed an erythematous reaction in two days, followed by the formation of papules and pustules. By about the fifth day the whole

area was covered with a creamy-white scab which, if removed at this stage, left a raw surface. Small nodules, 5 mm. in diameter, formed under the scab a few days later. These granulations were strawberry-like in appearance and resembled those seen on the legs in the natural disease. The experimental lesions healed in three to four weeks.

THE INFECTIVE AGENT

A suspension prepared from three-day-old rabbit lesions, and filtered through a gradocol membrane of APD 0·69μ, was infective for rabbits, but the incubation period was one to two days longer than that observed with unfiltered material. A Berkefeld V filtrate was non-infective.

Resistance to Chemicals

Infected scabs kept in buffered glycerol (equal parts of McIlvaine's buffer (*p*H 7·0) and glycerol) were viable when tested after 14 months' storage at 0 to 4° C. Treatment for five hours with ether, carried out according to the method described by Bennett, Horgan and Haseeb (1944), was found to inhibit the infective agent. This inhibitory effect of ether is in marked contrast to CPD virus which resists such treatment.

Microscopy and Electron Micrography

A large area on the abdomen of a lamb was infected with SFR, and scraped after four days. The material so obtained was suspended in buffer solution and centrifuged in horizontal and angle centrifuges, a procedure found to be satisfactory for the preparation of CPD elementary body suspensions. Smears of this material stained by Paschen's method were found to contain only vague particulate matter. Elementary bodies were not seen and repeated attempts failed to demonstrate them in preparations examined by the electron microscope. In these studies material from CPD lesions was examined at the same time with positive results.

Despite this failure to demonstrate elementary bodies, the fluid which gave these negative results was infective for rabbits and sheep, and when inoculated intradermally into rabbits it was possible to demonstrate a filamentous organism, morphologically resembling *Fusiformis necrophorus*, in the exudate from four-day lesions. It was not possible, however, to isolate the organism by cultural methods.

CROSS-IMMUNITY TESTS WITH CPD

In view of the resemblance between the clinical features of SFR and CPD, it was considered desirable to ascertain if any cross-protection existed between the two diseases, but unfortunately, it was only possible to carry out a limited number of experiments. The immunity of seven sheep which had recovered from CPD or simultaneous infection with CPD and SFR, was tested. The challenge

inoculum in the case of SFR consisted of a 1 in 100 suspension of dried goat scabs, administered by scarification on the flanks or thighs after removal of the wool. The CPD test material consisted of dilute suspensions of e.bs., which produced confluent lesions in controls.

TABLE I
CROSS-IMMUNITY TESTS WITH CPD AND SFR

Sheep No.	First infection		Interval between the two infections	Second infection		
	Infective agent(s) employed	Reaction		Type of reaction to :—		
				SFR	CPD (UF)	CPD (W)
			(Days)			
16	CPD (UF)	+++	164	++	—	—
15	„ (W)	+++	202	+++	+	—
13	„ (UF)	+++	251	+++	±	—
	SFR	+++				
8	CPD (UF)	+++	565	+++	+++	+++
20	Control			+++	+++	+++
20	CPD (UF)	+++	64	—	O	O
	„ (W)	+++				
	SFR	+++				
21	CPD (UF)	+++	32	±	O	O
11	„ „	+++	40	±	O	O
22	Control			+++	O	O

Strain UF = CPD (Glover, 1928).
W = A strain isolated from a human case of CPD at Woldingham in 1947.

Reaction +++ = Confluent eruptions.
++ = Discrete eruptions.
+ = Accelerated reaction, lesions healing in about nine days.
± = Thickening and redness of skin only (" vaccinoid " reaction)
— = No reaction.
O = Not tested.

The results of these experiments are given in Table I. It will be seen that sheep recently recovered from CPD or a simultaneous infection with CPD and SFR. were resistant to SFR, but those infected 164 days or more previously were susceptible. A sheep (No. 13) simultaneously infected with SFR and CPD proved fully susceptible to SFR 251 days afterwards, even though it was still strongly resistant to CPD.

It has been stated above that rabbits recovered from either SFR or CPD are not completely resistant to reinfection and that they develop accelerated reactions which are possibly an indication of partial immunity. They could not, therefore, be employed for critical cross-immunity tests. Two rabbits infected 16 and 20 days previously with CPD were, however, infected with SFR, and both developed a typical infection without any evidence of an acceleration reaction.

DISCUSSION

Strawberry foot rot, when transmitted to the rabbit, resembles CPD in its course, but the reaction is more marked and it also differs

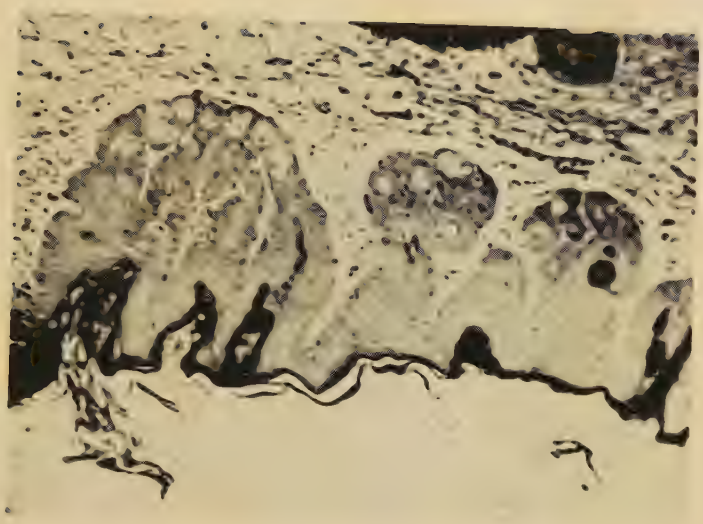

FIG. 1.—Four-day lesion, rabbit. Hyperplasia of epidermis. H. and E. × 120.

FIG. 2.—Six-day lesion, rabbit. Necrosis and infiltration of part of epidermis. H. and E. × 120.

in having a shorter incubation period. Histologically, the difference is even more apparent for, unlike CPD, the early response is more pronounced in the corium than in the epidermis and reticular degeneration is absent. Moreover, the necrosis and the proliferation of Malpighian cells is a more pronounced feature. In its host-range, SFR differs from CPD in that it is easily transmissible to the guinea-pig.

The infective agent has not been identified, but it is highly improbable that it is CPD virus or a virus of the pox group. This is evident from its easy inhibition by ether and the fact that it cannot be seen in electron micrographs of infective suspensions.

The immunity response to SFR is of some interest. In the rabbit it resembles CPD., but the absence of complete resistance which was observed in a recently recovered goat, is in marked contrast to the solid immunity arising from that disease (Blanc, 1943). While a satisfactory explanation cannot, at present, be offered for the resistance to SFR shown by sheep recently recovered from CPD, it is possible that the infective agent of the former has either some antigenic relationship with CPD virus or with one of the contaminants which invariably invade the CPD lesion. This immunity, however, is of comparatively shorter duration than that against CPD.

SUMMARY

Strawberry foot rot (SFR), a disease of sheep somewhat similar to contagious pustular dermatitis (CPD), has been transmitted to the rabbit, guinea-pig and the goat. The histopathology of the rabbit lesion has been described.

A limited number of tests revealed that the infective agent passes through gradocol membranes of 0·69μ average pore diameter, but cannot be demonstrated with the visual or electron microscopes. It is resistant to glycerol but is inactivated by ether.

Cross-immunity tests have shown that sheep recently recovered from CPD are resistant to SFR, but this immunity is more transitory than against CPD itself.

The foregoing studies indicate that the infective agent of SFR is different from that of CPD and probably does not belong to the pox group of viruses.

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